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TABLE OF CONTENTS

BROWMAN, LUDVIG G., Microphthalmia and maternal effect in the white rat 261
SMITH, WILLIAM K., Viability of interspecific hybrids in Melilotus .. 266
KIMURA, MOTOO, Process leading to quasi-fixation of genes in natural populations due to random fluctuation of selection intensities .. 280
RUBIN, B. A., The quantitative estimation of radiation induced mutations to streptomycin resistance in *Escherichia coli* 296
BREWBAKER, JAMES L., Incompatibility in autotetraploid *Trifolium repens* L. I. Competition and self-compatibility 307
WHITING, ANNA R., AND ROBERT C. VON BORSTEL, Dominant lethal and inactivation effects of nitrogen mustard on Habrobracon sperm 317
PITTENGER, THAD H., The general incidence of pseudo-wild types in *Neurospora crassa* 326
POMPER, S., K. M. DANIELS AND D. W. MCKEE, Genetic analysis of polyploid yeast 343
WOLFF, SHELDON, Some aspects of the chemical protection against radiation damage to *Vicia faba* chromosomes 356
SANDLER, L., AND G. BRAVER, The meiotic loss of unpaired chromosomes in *Drosophila melanogaster* 365
STEBBINS, G. L., AND ANTERO VAARAMA, Artificial and natural hybrids in the Gramineae, tribe Hordeae. VII. Hybrids and allopolyploids between *Elymus glaucus* and *Sitanion* spp. 378
RENDEL, JAN, A. NEIMANN SORENSEN AND M. R. IRWIN, Evidence for epistatic action of genes for antigenic substances in sheep .. 396
ALEXANDER, MARY L., Mutation rates at specific autosomal loci in the mature and immature germ cells of *Drosophila melanogaster* ... 409

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MICROPHTHALMIA AND MATERNAL EFFECT IN THE WHITE RAT

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Received August 21, 1953

MICROPHTHALMIA has been reported in a number of rat colonies (JONES 1925; KING 1931; HAIN 1936; BROWMAN 1941, 1943; QUISSENBERRY and BROWN 1942; CAMPBELL 1944). Published reports indicate that not only the embryological development but also the final eye condition may be quite varied in the several colonies (see BROWMAN and RAMSEY 1943 and CAMPBELL 1944).

The mode of inheritance of this character has not been satisfactorily worked out. Although the inheritance of certain kinds of microphthalmia (GRUNEBERG 1953a) and anophthalmia (CHASE and CHASE 1941; CHASE 1944) in the mouse seems to be fairly well understood, none of these explanations fits the data collected from the rat colony at Montana State University.

DESCRIPTION OF MICROPHTHALMIA

The development of eye lashes, sweat glands, oil glands, extrinsic ocular muscles, eye lids, lacrimal glands, and nasolacrimal glands is normal. However, the choroid, scleral, and lens material fail to develop normally. There is also a faulty development of the optic nerve and retinal tissue (BROWMAN and RAMSEY 1943). Ranges in eye development may include normal, almost normal, and various graded expressions down to a complete absence of macroscopic eyes. These varied conditions may be unilateral or bilateral with no consistent difference as to which side, or which sex is involved. Occasionally the optic nerve is missing or severely reduced in microphthalmic rats whose eyes are only slightly reduced in size. The general eye condition is not radically different from that described as " squint " in the mouse by BUTLER and ROBERTSON (1953).

CHASE and CHASE (1941) report an anophthalmic condition in mice which is not only eyeless but in which there are no ocular muscles nor associated eye structures.

RESULTS OF CROSSES

The maternal eye condition definitely influences the eye condition of the progeny (tables 1 and 2). When the maternal eye condition is microphthalmic the amount of microphthalmia in the hybrid young is significantly higher than in the hybrid young from normal colony females.

This maternal effect is lost in the second generation and the genetic constitution of the F_2 of the reciprocal crosses is expressed without significant modification from maternal influences.

Repeated backcrossing of phenotypically microphthalmic females to males of the normal strain for five successive generations reduced the percentage of microphthalmic young to less than 1% by the end of the fifth generation (a total of 1,252 animals). Similarly by repeated backcrossing of phenotypically normal eyed females to males from the microphthalmic strain for five successive generations it was possible to increase the percentage of microphthalmia at the end of the fifth backcross generation to over 75%. The *Bro* subline (normal eyes) had an increase from 0% to 78.2% microphthalmia involving

TABLE 1

Results of crosses involving a normal strain (N) and a microphthalmic strain (M). In each case the female parent is listed first and the phenotype is indicated by a subscript. The parents from the M strain were all phenotypically microphthalmic.

Cross	Offspring		
	Micro.	Normal	% Micro.
(1) $M \times M$	883	102	89.6
(2) $M \times N$	395	167	70.3
(3) $M \times (M \times N)_{nor}$	47	11	81.0
(4) $M \times (M \times N)_{mic}$	196	66	74.8
(5) $(M \times N)_{nor} \times M$	46	45	50.5
(6) $(M \times N)_{mic} \times M$	86	62	58.1
(7) $N \times (M \times N)_{nor}$	4	60	6.0
(8) $N \times (M \times N)_{mic}$	33	45	42.3
(9) $(M \times N)_{nor} \times N$	16	57	21.9
(10) $(M \times N)_{mic} \times N$	60	251	19.2
(11) $(M \times N)_{nor} \times (M \times N)_{nor}$	28	27	50.9
(12) $(M \times N)_{nor} \times (M \times N)_{mic}$	27	35	43.5
(13) $(M \times N)_{mic} \times (M \times N)_{nor}$	11	19	36.6
(14) $(M \times N)_{mic} \times (M \times N)_{mic}$	178	199	47.2
(15) $(F_2)_{nor} \times M$	123	40	73.6
(16) $(F_2)_{mic} \times M$	141	88	61.6
(17) $(F_2)_{nor} \times N$	47	169	21.8
(18) $(F_2)_{mic} \times N$	152	396	27.7
Totals	**2,473**	**1,839**	

a total of 1,236 animals in the five generations. The B-P-W subline (normal eyes) had an increase from 0% to 63.6% microphthalmia involving a total of 282 animals in the five backcross generations.

DISCUSSION

CHASE (1944) reported that anophthalmia in a strain of mice is dependent upon two pairs of genes inherited as independent duplicate factors. EATON (1937) suggested that microphthalmia in the guinea pig may be possibly dependent upon 2 factor pairs that are modified in their expression by the environment.

No simple 2- or 3-factor hypothesis satisfactorily explains the results reported here. There is a significant difference in the proportion of microphthalmia in the F_1 crosses from the two reciprocal crosses. When the carriers for the eye condition were introduced into the cross (micro × nor) by the homozygous microphthalmic females a high incidence (70%) of microphthalmia occurred in the F_1 progeny. When the carriers for microphthalmia were introduced via homozygous microphthalmic males (nor × micro) the incidence of microphthalmia in the F_1 was relatively low (20%). The progeny in the F_1 was definitely influenced toward the maternal characteristics.

TABLE 2

Results of crosses involving a normal strain (N) and a microphthalmic strain (M). In each case the female parent is listed first and the phenotype is indicated by a subscript. The parents from the M strain were all phenotypically microphthalmic.

Cross	Offspring		
	Micro.	Normal	% Micro.
(1) $N \times M$	128	494	20.6
(2) $M \times (N \times M)_{nor}$	33	15	68.8
(3) $M \times (N \times M)_{mic}$	57	10	85.0
(4) $(N \times M)_{nor} \times M$	228	127	64.2
(5) $(N \times M)_{mic} \times M$	11	11	50.0
(6) $(N \times M)_{nor} \times N$	44	121	26.3
(7) $(N \times M)_{mic} \times N$	34	25	57.6
(8) $N \times (N \times M)_{nor}$	33	189	14.8
(9) $N \times (N \times M)_{mic}$	9	37	19.6
(10) $(N \times M)_{nor} \times (N \times M)_{nor}$	229	293	43.8
(11) $(N \times M)_{nor} \times (N \times M)_{mic}$	29	21	58.0
(12) $(N \times M)_{mic} \times (N \times M)_{nor}$	5	12	29.4
(13) $(N \times M)_{mic} \times (N \times M)_{mic}$	16	20	44.4
(14) $(F_2)_{nor} \times M$	109	163	40.0
(15) $(F_2)_{mic} \times M$	28	11	71.8
(16) $(F_2)_{nor} \times N$	45	147	23.4
(17) $(F_2)_{mic} \times N$	29	155	15.7
Totals	1,067	1,851	

Upon inbreeding the F_1 progeny from both crosses the progeny indicated the same basic genetic constitution. There was no significant difference in the percentage of microphthalmia in the two reciprocal F_2 crosses (44.7% and 46.6%). Maternal influence of the parent generation was not evident in the F_2 generation.

FORTUYN (1931) said that 2 strains of mice differed in the number of tail rings, and the 2 reciprocal hybrid progeny were not intermediate nor alike, but that each deviated to its maternal side. "The mother having the highest tail ring number always produces offspring having the higher tail ring number," and "a definite maternal effect was patent as regards the inheritance

of tail ring number" (LAW 1938). GREEN and RUSSELL (1951) also indicated that hybrid mice of similar genetic constitution but of dissimilar skeletal type tend to resemble the strain of the maternal parent.

Cytoplasmic factors of the egg are not involved in many maternal effects, for RUNNER (1951) demonstrated that survival rates of transplanted genetically tagged ova were influenced by maternal factors which effectively restricted the number of young in certain female mice. Litter size, position in the uterus, crown-rump presentation, right-left side, the particular uterine horn, and serial order in the uterine horn exerted no significant effect on variation of skeletal type in mice (GREEN and GREEN 1946a).

GRUNEBERG (1953b) in analyzing the genetical behavior of a number of skeletal characters in mice suggests that although a physiological difference (maternal effect) may influence the manifestation of one character it need not necessarily affect other potentially responsive characters. He further suggests that certain entities are "quasi-continuous" in the sense that the underlying genetic basis is a continuous variable with multiple factor inheritance which is divided by a physiological threshold into normal and abnormal animals. The abnormality is usually capable of graded expression. Microphthalmia in over 9,000 rats of the M.S.U. rat colony in this study seems to fit generally into this suggestion.

SUMMARY

1. Crossing female rats from a strain breeding true for microphthalmia with homozygous normal-eyed males produces a progeny with a high percentage of microphthalmia. The reciprocal cross of homozygous normal females mated with homozygous microphthalmic males yields a progeny with a very low percentage of microphthalmia. This evidence is interpreted to indicate a maternal effect on developing embryos, presumably of the same genetic constitution, with the development of the young definitely influenced toward the maternal characteristics.

2. Inbreeding of the F_1's and reciprocal F_1's yields progeny which do not differ significantly from each other in the degree or percentage of microphthalmia. The maternal effect does not persist beyond the first generation.

3. Microphthalmia in this strain of rats is believed to be the result of polygenic factors with incomplete penetrance or possibly quasi-continuous inheritance, the genetic factors being modified in their expression by maternal influences.

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VIABILITY OF INTERSPECIFIC HYBRIDS IN MELILOTUS[1]

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Received September 5, 1953

THE cultivated forms of sweetclover (*Melilotus spp.*) have a high content of a bitter substance, coumarin. In the search for non-bitter strains, BRINK (1934a) reported the finding of a non-bitter strain which was later identified as an annual form of the typically biennial *Melilotus dentata*. When this strain was crossed with *M. alba*, the F_1 hybrids proved to be chlorophyll-deficient (BRINK unpublished; STEVENSON and WHITE 1940) and died shortly after they appeared above ground. Hybrids have now been reared to maturity by grafting on common sweetclover (SMITH 1943). The inability of the F_1 hybrids to survive under natural conditions beyond the cotyledonary stage constitutes an effective barrier against the exchange of genes between the two species. It has, therefore, been of interest to explore interspecific relationships in the genus beyond the *alba* × *dentata* cross to determine whether the same or a similar barrier exists between other pairs of species. During the past several years, pollinations have been made between pairs of species within the subgenus Eumelilotus to test compatibility between the species, and to determine the prevalence of the chlorophyll-defective character among the hybrids obtained. The results are considered in the present paper.

Several interspecific hybrids in addition to *M. alba* × *dentata* have been reported in Melilotus. Normal green hybrids are readily obtained in crosses between *M. alba* and *M. suaveolens* (STEVENSON and KIRK 1935; JOHNSON 1942; WEBSTER 1950). Cross pollinations among 13 sweetclover species were made by MENDOZA (1946). Seeds obtained were planted by WEBSTER (1950) and normal green hybrids were obtained in the cross *M. alba* × *polonica* as well as in *M. polonica* × *suaveolens*. Four hybrids were also obtained in the cross *M. italica* × *messanensis*. From *M. suaveolens* × *wolgica*, a hybrid, light green in color, grew to produce flowers and a few fruits. A weak, light green hybrid that did not set fruits was also obtained in the cross *M. alba* × *taurica*.

STOCKS USED

Crosses were made between species within the subgenus Eumelilotus (SCHULZ 1901), the section to which with one exception the species found in cultivation belong. SCHULZ describes eleven species in Eumelilotus but it

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was not possible to obtain seed of three of them, *M. hirsutus* Lipsky, *M. Kotschyi* Schulz and *M. Urbanii* Schulz. The eight species included in the present study are listed in table 1. In five of the eight species, more than one collection or introduction was used.

TABLE 1

Species and strains of Eumelilotus included in the study of interspecific hybridization.

Species and strain	Number	Origin or description
M. alba		
(a)	Ac 15	Introduction from Rostov-on-Don, U.S.S.R.
(b)	Ac 34	Down Dwarf, selected by E. E. Down, Mich.
(c)	Ac 62	Received as *M. bicolor*
(d)	Ac 209	"Pioneer" strain, selected at Saskatoon, Sask., Canada
(e)	C 231	Fowlds Fine Stem, selected by M. Fowlds, S. Dakota
(f)	O 114-4	Iowa late white
(g)	O 199-18	Commercial common white
M. altissima		
(a)	Ac 158	04389
(b)	Ac 165	P.I. 29,831
(c)	B 130	Received from H. B. Sifton, Toronto, Canada
M. dentata		
(a)	Ac 72	Annual collected by Dorsett and Morse near Peiping, China
(b)	Ac 91	Saratov, U.S.S.R.
(c)	Ac 92	West Siberia, U.S.S.R.
(d)	Ac 98	Annual collected by Prof. Anton Fröhlich, Nikolsburg, Czechoslovakia
M. officinalis		
(a)	4-33-33	Selected by R. A. Brink, Wisconsin
(b)		Commercial common yellow
M. polonica		
	Ac 141	P.I. 108,647
M. suaveolens		
	Ac 35	Peiping, China
M. taurica		
(a)	Ac 151	P.I. 67,854
(b)	Ac 152	P.I. 116,709
M. wolgica		
	Ac 163	P.I. 27,292

METHODS

All artificial crosses were made in the greenhouse during the winter and spring months when natural daylight was supplemented with mazda lamps to give approximately an 18-hour photoperiod. In making each cross, the petals were removed from recently opened flowers and the pollen removed by suction developed from a small vacuum pump. Pollen of the staminate parent was ap-

TABLE 2

Plumpness of seed and color of seedlings in crosses between M. alba, M. dentata, M. taurica and M. altissima.

Cross	No. of flowers	No. of pods*	No. of seeds				Seedlings			
								Hybrids		
									Emerged	
			Plump	Shrunken	Much shrunken	Brown aborted	No. selfs	No. not emerged	No.	Color class
M. alba × *dentata*	335	109	40				33	2	5	(1)
				59				20	36	(1)
					39			27	12	(1)
						21				
M. alba × *taurica*	14	2	2†				1		1	(2)
M. taurica × *alba*	255	50n	51				47	4		
				1					1	(2)
						9				
		115sm		13			1	3	9	(2)
						114				
M. alba × *altissima*			30†				21	8	1	(2)
M. altissima × *alba* '43			37†				7	16	13	(2)
M. altissima × *alba* '44	11	8n		6				3	3	(2)
						3				
		2sm		1					1	?
						1				
M. taurica × *dentata* '43	35	8n	8†				4	1	3	(5)
		2sm				2				
M. taurica × *dentata* '44	44	21	11				11			
				7			1		6	(5)
						3				
M. altissima × *dentata*	43	8	7				7			
					4			4		
M. dentata × *altissima*	20	7	7				7			
					1			1		
M. altissima × *taurica*	33	21	12				12			
				5				2	3	?
					4			4		

*n—normal size; sm—much smaller than normal.
†Seeds not classified for degree of shriveling.

plied without delay to the stigma of the pistillate parent with a toothpick tipped with fine emery cloth.

Favorable conditions for germination, including scratching of individual seeds with a razor blade and planting at a shallow but uniform depth, were provided for the seeds in the greenhouse. Under these conditions, practically every seed of the parent species gave rise to a thrifty seedling. Consequently, in summarizing the results obtained from crosses between species, seeds from which no seedling emerged were classed as hybrid. There may be some error arising from ineffective scarification, the accidental omission of scarification or the death of selfed seedlings before emergence, but the error is likely to be small.

After the cotyledons were fully expanded, the seedlings were rated for the intensity of green color on a scale from 1 to 6 where 1 represents a seedling almost devoid of chlorophyll and 6 a normal green seedling.

RESULTS

The mature seeds obtained from crosses between species were classified as plump, shrunken or much shrunken and the number of seedlings arising from each class of seed was recorded. In addition, in certain crosses there were aborted seeds that were small, much shrunken, brown in color and non-viable. Presumably fertilization occurred but the embryos died at an early stage in development. These aborted seeds were very similar to those described by STEVENSON and KIRK (1935) as occurring in the cross between *M. alba* and *M. officinalis*. THOMPSON (STEVENSON and KIRK 1935) found that fertilization apparently occurs normally in crosses between *M. alba* and *M. officinalis* but the hybrid embryo grows slowly and dies after reaching a length of about 0.5 mm.

Attention was given first to the *alba* × *dentata* cross (table 2). The 21 brown, aborted seeds were sharply distinguished from the seeds that proceeded to maturity. The mature seeds ranged from plump to much shrunken but seeds in the much shrunken group were substantially larger than the aborted structures and were olive to brownish green in color rather than dark brown. The 40 seeds classed as plump gave 33 seedlings like the *alba* parent—from accidental self-pollination—while five gave chlorophyll-deficient hybrids and two did not emerge as seedlings. In contrast, from the 59 shrunken seeds, no *alba* plants were obtained but 36 proved to be hybrid and 20 did not emerge as seedlings; three were not planted. Twelve of the 39 much shrunken seeds gave hybrids and 27 did not emerge; the proportion of seeds failing to emerge is, as might be expected, somewhat higher in this much shrunken group. As already mentioned, seeds not emerging are probably hybrid and, if in this cross they are added to those giving chlorophyll-deficient seedlings, only 7 of the total 102 hybrid seeds were not classified as in some degree shrunken. There is, therefore, a wide variation in the degree of development of the hybrid seeds and few, if any, are as plump as those of either parent.

The F_1 seedlings from this cross were described previously (SMITH 1943). They ranged from practically colorless to pale yellowish green, and usually

received a rating of 1 although some were class 2. A proportion of the seedlings, especially those arising from much shrunken seeds, were smaller than normal and were very weak, the cotyledons remaining encased in the seed coat after appearing above ground. If left to grow in the soil, none survived beyond the early seedling stage. In some instances the unifoliolate leaf had attained a length of 2–3 mm before the seedling ceased growth and collapsed.

Pods harvested after interpollination of *M. alba* and *M. taurica* (table 2) could be sorted into two sizes, normal (n) and small (sm). The normal pods gave without exception plump seeds and as the seedlings from the plump seeds emerged, they were found to be accidental selfs. Four not emerging were obtained from the pollination of one inflorescence and constituted one lot in a planting. It is probable that in this lot scarification of the seeds had been accidentally omitted and the seeds should be included with the 47 seeds that yielded typical *taurica* plants. The small pods proved to be the most fruitful source of hybrids. Of the 13 shrunken seeds derived from the small pods, 12 were of a dilute olive-green color instead of the normal olive-yellow and all proved to be hybrid. The remaining shrunken seed was green in color and gave rise to a typical *taurica* plant. It may have been harvested accidentally before the seed was ripe. In this cross, the rate of seed abortion is very high.

The F_1 hybrids of *M. taurica* × *alba* and the reciprocal showed somewhat more green color that the *alba* × *dentata* hybrids, all being assigned to color class 2. None survived to show true leaves and consequently they had much less pigment than the light green hybrid reported by WEBSTER (1950) as growing for two years and producing a few flowers. In the tests reported here, only one strain of *M. taurica,* Ac 152, was used in combination with three strains of *M. alba.*

Reciprocal pollinations between *M. alba* and a yellow-flowered species *M. altissima* also gave chlorophyll-deficient hybrids having a color reading of 2 (table 2). The hybrid seedlings were vigorous and made good growth when grafted on common sweetclover.

The occurrence of very pale hybrids in crosses of *M. alba* with any of the three species *M. dentata, M. taurica* or *M. altissima* suggested that the three species might be similar in genes for chlorophyll development and on intercrossing might yield normal green hybrids. Several interpollinations were made. The cotyledons of the nine *taurica* × *dentata* hybrids (table 2) were nearly normal green, being rated when fully expanded as class 5 on the color scale 1 to 6. The seedlings were vigorous, being almost as strong as the *taurica* seedlings and larger than those of *M. dentata.* Although the cotyledons were green, the unifoliolate leaf appeared to be devoid of chlorophyll or yellow pigments as it unfolded so that both blade and petiole were of a rose pink color, presumably because of the presence of anthocyanin pigment. As growth progressed, the leaves gained in chlorophyll content. When the plant reached the flowering stage, the color of the older leaves was approximately normal green. The hybrids made a vigorous growth and flowered profusely. One of the hybrids, after forming seed in the greenhouse, was held during the summer

in a cold frame. In the autumn, well-developed buds were noted at the crown. The young shoots from these crown buds in the greenhouse had at first only a trace of green pigment and were of a rose pink color similar to that of the young unifoliolate leaf. Again as the shoots developed, green color deepened and mature leaves were normal green at flowering. But the young leaves at the flowering stage were deficient in chlorophyll and as they expanded were pale green, corresponding to " lettuce green " (RIDGWAY 1912); as they matured, they became normal green.

The contrast between the very pale hybrids arising from the crosses of *M. alba* with both *M. dentata* and *M. taurica* and the nearly normal foliage in the *taurica* × *dentata* hybrids suggests that the difference between the two latter species in the genes for chlorophyll development is less than that between *M. alba* and *M. dentata* or *M. alba* and *M. taurica.*

Crosses were also made reciprocally between *M. altissima* and *M. dentata,* but no hybrid seedlings were obtained. Five shrunken seeds may have resulted from cross pollination but all five were much shrunken; three were greenish brown in color and two were very small and dark green. In the *altissima* × *taurica* cross, nine seeds were obtained in addition to the 12 seeds resulting from accidental self-pollination of the *altissima* parent. All nine presumably hybrid seeds had a greenish tint and when planted only three pushed the cotyledons above ground. The cotyledons were tightly enclosed in the seed coat and the seedlings were so weak that it was not possible to remove the seed coat without injury to the hypocotyl. Consequently, the color of the cotyledons in this cross could not be determined.

An additional group of interspecific crosses within Eumelilotus is included in table 3. When *M. alba* was the pistillate parent in the *alba* × *wolgica* cross, three chlorophyll-deficient hybrids were obtained and all showed only a trace of chlorophyll giving a reading of 1. It was noted that the seedlings showed very little yellow color, making them almost white. The seedlings were very weak. In the reciprocal cross, one hybrid seedling was more vigorous than the reciprocal hybrids but the chlorophyll reading was no higher than 1. A second hybrid was markedly different in appearance. It was chlorophyll-deficient but had much more chlorophyll than the other hybrids, receiving a color rating of 4. The basis for the appearance of a seedling with substantially more green color than the other four is not clear. The plant recorded as the pistillate parent of the aberrant seedling was *M. wolgica* and the pollen parent a strain of *M. alba* carrying a marker consisting of a protuberance on the underside of the cotyledons. The aberrant seedling showed this marker and might therefore be considered clearly a cross since the marker has not been noted in the *wolgica* species. However, the plant may have been a stray seedling since, during the same season, progenies from the cross *M. alba* × *dentata* were being grown from plants heterozygous for the marker and for genes determining reduction in chlorophyll content. The seedling was grafted on common sweetclover but died after the development of a small first true leaf and further evidence as to its origin could not be obtained.

TABLE 3

Plumpness of seed and color of seedlings in crosses between pairs of seven species in the subgenus Eumelilotus.

Cross	No. of flowers	No. of pods*	No. of seeds				Seedlings				
								Hybrids			
										Pale	
			Plump	Shrunken	Much shrunken	Brown aborted	No. selfs	No. not emerged	No. green	No.	Color class
M. alba × *wolgica*	20	4	6				3			3	(1)
M. wolgica × *alba*	49	11	12				8	2		2	(1,4)
						2					
M. wolgica × *dentata*	40	15	4				4				
				10				6		4	(3)
						1					
M. wolgica × *altissima*	12	6	7				6			1	?
M. altissima × *wolgica*	14	3	3				1	1		1	(3)
M. alba × *polonica*	69	25	26				6	1	19		
M. polonica × *alba*	12	7	3						3		
				4				4			
M. alba (b) × *suaveolens*	44	17	6				6				
						21					
M. alba (e) × *suaveolens*	19	10	14				9		5		
M. suaveolens × *alba* (c)	44	22		2					2?		
						15					
M. suaveolens × *altissima*	54	18n	12				11				
						8					
		9sm				9					
M. suaveolens × *dentata* (a)	30	11var	2				2				
						9					
M. suaveolens × *dentata* (b)	17	7			2			2			
						5					
M. suaveolens × *dentata* (c)	16	5	1				1				
						7					
M. suaveolens × *dentata* (d)			4†				2			2	(1)
M. suaveolens × *taurica*	27	2n	1				1				
					1			1			
		19sm	1				1				
						27					

*n—normal size; sm—small; var—variable in size. †Seeds not classified for degree of shriveling.

M. wolgica was next combined with two other species shown to give hybrids almost devoid of chlorophyll in crosses with *M. alba*. Using *M. wolgica* as the pistillate parent with *dentata* pollen, ten seeds smaller in size and slightly less plump than *wolgica* seeds were obtained (table 3). Only four emerged as seedlings when planted and all proved to be vigorous hybrids with substantially more chlorophyll pigment (color class 3) than the seedlings in the *alba* × *wolgica* cross. On one hybrid reared as a graft, the color of the leaves at flowering time corresponded to " lettuce green " (RIDGWAY 1912). This is a green-green-yellow and only slightly less green that the *wolgica* or *dentata* parents. In the *wolgica* × *altissima* cross, a probable hybrid with less than the normal amount of chlorophyll was obtained but the cotyledons were damaged by disease before a reliable reading was obtained. The reciprocal cross, *M. altissima* × *wolgica*, gave a vigorous hybrid of color class 3. After growing as a graft for a month, its color was rated as 4 +. Thus the hybrids obtained when *M. wolgica* was crossed with either *M. dentata* or *M. altissima* have a higher level of chlorophyll than the hybrids resulting from crosses of any of the three species with *M. alba*.

The first interspecific hybrid between *M. polonica* and *M. alba* resulted from a natural cross occurring in a nursery at Madison in which the two species were growing side by side and in flower after other species in the nursery had ceased flowering. Subsequently, hybrids were obtained from crosses in the greenhouse (table 3). All hybrids are normal green. Hybrid seeds from the use of *M. polonica* as the pistillate parent tended to be more shriveled than seeds from the reciprocal cross.

The fact that *M. alba* and *M. suaveolens* can be crossed readily and give normal green hybrids has been recognized for some time (STEVENSON and KIRK 1935; JOHNSON 1942). JOHNSON reported only mature seeds in his crosses although he found hybrid seeds to be only about half the weight of the seeds of the parent. STEVENSON and KIRK, however, found that while some inbred lines of the *alba* variety produced only normal seeds in crosses with Redfield Yellow (later shown to be a strain of *M. suaveolens*), in crosses between other lines of *M. alba* and the same strain of *M. suaveolens*, a proportion up to 37 percent of the flowers pollinated gave abortive embryos. In the present studies after the transfer of pollen from a Peiping strain of *M. suaveolens* to the Down Dwarf strain of *M. alba*, only brown aborted seeds were found in addition to the six plump seeds that proved to have resulted from selfing (table 3). In contrast, no aborted and five hybrid seeds were obtained from the 10 pods resulting from the application of *suaveolens* pollen on the *alba* strain, Fowlds Fine Stem. Again the same strain of *M. suaveolens* as the pistillate parent in a cross with a third strain of *M. alba*, Ac 62, gave 15 brown abortive seeds but they were larger than those in which embryos cease growth at approximately 10 days after pollination. Two seeds were small and green; when planted, two small, weak but normal green seedlings arose. The seedlings did not survive until flowering. They were probably hybrid although in the absence of flowers this could not be determined precisely. The stocks involved

in the hybridization between the two species appear to offer suitable material for a study of the genetic basis for the abortion of hybrid seeds—a barrier between species but one different in character from that of chlorophyll deficiency.

Crossing between *M. suaveolens* and *M. altissima* has been attempted on a very limited scale but no mature hybrid seeds have been obtained (table 3). A few crosses were made between *M. suaveolens* and four quite different strains of *M. dentata.* Only in the cross with the annual Ac 98 *dentata* strain were two hybrid seedlings obtained and they were very pale with a reading of 1. The cross with *M. dentata* Ac 91 gave two much shrunken seeds that did not produce seedlings. They were probably weak hybrids. Several flowers on one plant of *M. suaveolens* were pollinated with pollen from a plant of *M. taurica.* One shriveled seed did not give a seedling while 27 aborted seeds were obtained from 19 immature pods. The appearance of a seed with better development than the aborted seeds suggests that further crossing may give rise to viable seeds.

The finding of sweetclover plants with pale yellow flowers has been reported and natural crossing between the yellow-flowered *M. officinalis* and the white-flowered *M. alba* has been suggested. STEVENSON and KIRK (1935) made an extensive series of crosses under greenhouse conditions between *M. alba* and *M. officinalis* but no viable hybrids resulted. The seeds obtained were smaller than normal, were shrunken and of a greenish-brown color. In the present studies, several *alba* × *officinalis* crosses were made and the results were the same as those reported by STEVENSON and KIRK. These investigators also pointed out that the supposed hybrids reported by earlier workers were not obtained under controlled conditions and since crosses can readily be made between *M. alba* and the yellow-flowered *M. suaveolens* resulting in plants with cream-colored flowers, *M. suaveolens* is probably the species involved rather than *M. officinalis.*

A few crosses were also made between *M. officinalis* and each of the species *M. altissima, M. dentata, M. suaveolens, M. taurica* and *M. wolgica* but hybrid seedlings have not been obtained. STEVENSON and WHITE (1940) reported that in pollinations between *M. dentata* and *M. alba, M. suaveolens* and *M. officinalis,* respectively, no seedlings survived beyond three weeks. It was not stated specifically whether seedlings were obtained in the *officinalis* × *dentata* cross. The present results point to *M. officinalis* as the only one of the eight species which did not give seedlings in crosses with at least one of the other seven. However, the number of flowers pollinated was not large, and usually only one strain of each species was tested.

Intercompatibility of the eight species

The results of the crosses between species within the subgenus Eumelilotus, already described for each attempted cross, may now be summarized (table 4). This will reveal more clearly the relationships among the species considered.

The eight species appear to fall into three groups on the basis of the type

of hybrid obtained. Group A includes *M. alba, M. polonica* and *M. suaveolens.* Except for aborted seeds when certain strains are used in the crosses between *M. alba* and *M. suaveolens,* intercrossing among the three species yields only normal green hybrids (table 3). WEBSTER (1950) also reported deriving normal green hybrids in the *polonica* × *suaveolens* cross. Further, the three species in group A, as far as tested, do not give normal green hybrids when crossed with species in group B (*M. altissima, M. dentata, M. taurica* and *M. wolgica*). The crosses *M. suaveolens* × *altissima* and *M. suaveolens* × *taurica* have given only aborted seeds but where hybrid seedlings have been obtained, the green color has been no more intense than class 2. One seedling of color class

TABLE 4

Intercompatibility relationships of eight species of Eumelilotus.

Female parent	Male parent: *M. alba*	*M. polonica*	*M. suaveolens*	*M. altissima*	*M. dentata*	*M. taurica*	*M. wolgica*	*M. officinalis*
GROUP A								
M. alba		(6)*	(6)	(2)	(1)	(2)	(1)	un†
M. polonica	(6)		(6)‡					
M. suaveolens	(6)	(6)‡		un	(1)	un		un
GROUP B								
M. altissima	(2)				un	?	(3)	un
M. dentata				un				un
M. taurica	(2)				(5)			
M. wolgica	(1,4)			?	(3)			
GROUP C								
M. officinalis	un		un			un	un	

*Numbers in parentheses indicate color of seedlings on a scale: 1—very pale, 6—normal green.

†un signifies that pollinations were made but no hybrid seedlings were obtained.

‡Reported by WEBSTER (1950).

4, possibly a stray, was noted in the *alba* × *wolgica* cross. When species of group B are intercrossed, the hybrids obtained have had less chlorophyll than the normal but where readings could be made, they have had more chlorophyll than hybrids between species of group A with species of group B. Color readings on the cotyledons have been either class 3 or class 5.

No hybrid seeds have been obtained in combinations involving *M. officinalis.* JOHNSON (1942) obtained no mature seed in crosses with *M. suaveolens,* and STEVENSON and KIRK (1935) obtained only abortive seeds from matings with both *M. alba* and *M. suaveolens.* In the present studies, crosses have been attempted between this species and six other species. Interpollinations between *M. officinalis* and *M. alba* have yielded only abortive seeds as earlier workers found. Likewise, no seeds have been obtained in crosses between *M. officinalis*

and the four species here listed in group B. *M. officinalis* at present, therefore, is assigned to group C.

DISCUSSION

Interspecific hybrids are not readily obtained in genera of the Leguminosae. In Lathyrus, SENN (1938) tried 69 interspecific combinations among seventeen species but there was not a single viable hybrid. Interspecific matings have been extensively tested in two genera closely related to Melilotus, Medicago (OLDEMEYER 1951) and Trifolium (GURAVICH 1950), but few interspecific hybrids were found. In Eumelilotus, however, normal green hybrids were obtained in crosses among the three species, *M. alba, M. suaveolens* and *M. polonica* (group A) in each combination tested. Hybrids, although chlorophyll-deficient, resulted from crosses between species of group A with species of group B; and some combinations within group B gave pale hybrids.

An unusual feature in the results of the interspecific crosses is the high proportion of matings giving chlorophyll-deficient hybrids, in contrast to results with related genera in which any hybrids obtained have been normal green. Instances of a deficiency of chlorophyll being the primary cause of lethality in the first generation of an interspecific cross are rare. Frequently F_1 seedlings are weak and fail to survive to maturity. Occasionally, deficiency in chlorophyll is mentioned but appears to be associated with very weak growth (MANGELSDORF and EAST 1927; LAIBACH 1930). In several instances as in Oenothera (SCHWEMMLE and ZINTL 1939) evidence has pointed to failure of plastids of one species to function normally along with genes from the other species. However, in the lilac genus many of the crosses produce viable seeds but the seedlings are albinos and soon die (SAX 1945).

Lack of chlorophyll in the F_1 seedlings constitutes an effective interruption in the reproductive cycle but a break at an earlier stage in the cycle is also noted in Melilotus. In the *alba* × *officinalis* cross, only dark-brown aborted structures were obtained and no mature seeds. A few chlorophyll-deficient hybrids were obtained in *M. alba* × *taurica* but a large proportion of the matings resulted in dark-brown aborted seeds. THOMPSON (STEVENSON and KIRK 1935) found that in the *alba* × *officinalis* cross the endosperm makes little growth after 10 days but the embryo continues growth, although slowly, until about 17 days after pollination. No attempt was made in the crosses giving aborted seeds to trace the development of the several tissues within the ovule and thus extend beyond the *alba* × *officinalis* cross the observations of THOMPSON. Several crosses yielded a proportion of shrunken seeds, some failing to germinate but others giving weak seedlings.

Only a beginning has been made in the study of the genetic basis for lethality in the F_1 hybrids and this only for chlorophyll deficiency in the cross *M. alba* × *dentata*. The problem cannot be approached directly because the chlorophyll-deficient hybrids reared by grafting on normal sweetclover do not set seed on selfing. A few seeds are obtained by backcrossing to the parent species (SMITH 1943; BRINGHURST 1951). From a study of backcross progenies in the *alba* × *dentata* cross, BRINGHURST has shown the presence of a

series of more than three non-allelic genes each of which in homozygous condition gives a plant with a sharp reduction in chlorophyll pigment. While some heterozygotes singly cannot be distinguished from normal green plants, dominance is incomplete in certain heterozygotes. When two or more genes are present in the heterozygous condition, a marked reduction in chlorophyll content is observed. However, the mechanism by which these genes in certain genotypes are accompanied by a reduction in chlorophyll but show no effect in the parent species has not been determined. The genetic basis for inviability of the F_1 hybrids between species has now been determined in certain genera. Two dominant complementary genes were found in Streptocarpus (LAWRENCE 1947) while STEPHENS (1950) advances evidence of pseudoallelism in the "corky" hybrids in cotton and suggests that other examples of hybrid weakness may have a similar basis.

The widespread occurrence of the lethal chlorophyll-deficiency in interspecific hybrids in Melilotus and its absence in related genera might suggest that the species combined in group A or group B on the basis of a similar response in interspecific crossing are also similar in other characteristics and presumably also more closely related. Examination of the eight species gives little support to this suggestion. All eight species have the same chromosome number (DARLINGTON and JANAKI AMMAL 1945; WIPF 1939), and all are included in Coelorytis, one of the five sections of the genus Melilotus (SCHULZ 1901). Two of the three species in group A, *M. alba* and *M. suaveolens,* are very similar in morphology, differing mainly in flower color and dentation of the leaflets. But in crosses the hybrid seeds weigh only about half those of the parent species (JOHNSON 1942) and the F_1 hybrids show a substantial proportion of aborted pollen grains frequently exceeding 50 percent (WEBSTER 1950). Also, in the present studies, crosses involving certain strains gave only aborting embryos. The third species in this group, *M. polonica,* is sharply distinguished morphologically from the other two. The species of group B, the four species that give chlorophyll-deficient hybrids in crosses with *M. alba,* are well-defined species, clearly distinct from one another in morphological characters. In addition, *M. dentata* differs from the other three in being free of coumarin (BRINK and ROBERTS 1937) and in a more pronounced tendency towards spontaneous self-pollination. The remaining species, *M. officinalis,* has not yielded hybrids when crosses were made with the other seven species. Thus no conclusion can be reached regarding similarities or differences between genes for chlorophyll development in this species and those in the other seven species. *M. officinalis* differs from the other seven mainly in the 6-ovule ovary (SCHULZ 1901) and high frequency of self-incompatibility (KIRK and STEVENSON 1931; BRINK 1934b; SANDAL and JOHNSON 1953). Additional information on meiotic and genetic behavior in various interspecific hybrids in this portion of the genus will provide a more precise picture of relationships between the species, but from the present available evidence the divergence between the three groups does not appear to be significantly greater than that apparent between species within either group A or group B.

The barriers of hybrid embryo abortion and chlorophyll-deficiency in F_1

may have played a part in the evolution of species in the genus but the participation of other agencies in blocking or hindering the interchange of genes between species is suggested. The cross *M. alba* × *dentata*, now studied in more detail than any other, provides a good illustration. The two species *M. alba* and *M. dentata* are adapted to sharply contrasted environments—*M. alba* to upland meadows and *M. dentata* to salty soils and brackish marshy areas. Nearly all strains of *M. dentata* set seed spontaneously without tripping of the flowers by insects, and fertilization takes place soon after the petals unfold, thereby reducing the amount of cross pollination. When crosses are made between the two species (table 2), a proportion of the embryos abort but other embryos develop to give shrunken seeds and only a small proportion result in plump seeds. Low vitality is noted in the hybrid seed and many of the shrunken seeds fail to develop into seedlings. Further, hybrids reared as grafts on normal green sweetclover plants have up to 80 percent of poor pollen and fail to set seed on self-pollination. Thus the two species are separated by a number of barriers, several of which alone could effectively prevent exchange of genes between the two species.

SUMMARY

Pollinations between pairs among eight species in the subgenus Eumelilotus reveal three groups of species on the basis of the type of hybrid obtained. Crosses between any member of group A (*M. alba, M. suaveolens, M. polonica*) and a member of group B (*M. altissima, M. dentata, M. taurica, M. wolgica*) gave hybrid seedlings that were almost devoid of chlorophyll and lived for only a few days unless grafted on normal green sweetclover plants. Crosses between pairs within group A gave normal green hybrids; crosses within group B resulted in hybrids that were chlorophyll-defective but showed more pigment than hybrids between A and B. The chlorophyll-deficient character, which is an effective barrier in nature between the species, is considered in its possible significance for the evolution of species in the subgenus.

The reproductive cycle was also interrupted at an earlier stage in these crosses, resulting in dark-brown aborted seeds. Shriveled seeds were also obtained that failed to germinate or gave weak hybrids. No mature seeds were obtained from crosses between *M. officinalis* and any one of the six species tested. This species, therefore, cannot be assigned to either of the two groups A or B and is placed in group C.

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PROCESS LEADING TO QUASI-FIXATION OF GENES IN NATURAL POPULATIONS DUE TO RANDOM FLUCTUATION OF SELECTION INTENSITIES * [1]

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AMONG factors that may produce random fluctuation of gene frequencies in natural populations, random sampling of gametes and random fluctuation of selection intensities may be especially important in relation to evolution. On the process of " random drift " that will be realized in finite populations due to the random sampling of gametes in reproduction, not only precise mathematical studies have been carried out (WRIGHT 1931, 1945; FISHER 1930; HALDANE 1939; FELLER 1950) but also several model experiments have been undertaken (cf. HOUSE 1953). Since 1931, WRIGHT has repeatedly emphasized the evolutionary significance of random drift in a natural population which is subdivided into many partially isolated sub-groups. His theory is now accepted by many evolutionists such as HALDANE (1949), MULLER (1949), DOBZHANSKY (1951) and others.

On the other hand, no special attention seems to have been paid to the random fluctuation of gene frequencies due to the random fluctuation in the selection intensities until FISHER and FORD (1947) emphasized its prevalence in natural populations and challenged the theory of Wright by denying any significance of random drift due to small population number in evolution. This led to a polemic (cf. WRIGHT 1948; FISHER and FORD 1950; WRIGHT 1951). Experimental studies on natural populations have been carried out by the school of FISHER and FORD (e.g., SHEPPARD 1951; DOWDESWELL and FORD 1952) and by LAMOTTE (1952).

In spite of these, no mathematical investigations seem to have been worked up on the process of change due to the random fluctuation of selection intensities, except a short article reported by the present author (KIMURA 1952a), though WRIGHT (1948) gave a distribution of gene frequencies in steady state for a special case.

In his report, the present author proved, using a method of transformation and approximation, that the process can be regarded as a deformed Gaussian process. In the present paper, a pair of alleles lacking dominance will be assumed. The process of change of their frequencies when their selection coefficients fluctuate fortuitously from generation to generation around a mean value

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0 is simplest for mathematical treatment. Investigation of this process is a main subject of this paper. The process of change which will be found in terminal portions of the frequency distribution curve is especially important in this connection, so that a precise analysis of it will be undertaken. Through this analysis the reader will be led to new concepts of " quasi-fixation " and " quasi-loss " of an allele. Comparison of this process with that of random drift due to small population number is another important subject in the present report. Though there are many theoretical studies on the process of random drift, they are usually restricted to the state that will be realized after a sufficient number of generations. In that state the distribution curve assumes a fixed form and the probabilities of all heterallelic classes decrease at a constant rate of 1/2N, with fixation and loss of the gene occurring at the same rate.

Hence more extensive studies may be needed to make such a comparison. In the present paper an asymptotic solution for the process of random drift due to small population number will be presented for the first time.

PROCESS OF CHANGE OF FREQUENCIES OF ALLELES WHICH ARE NEUTRAL ON THE AVERAGE AND LACKING DOMINANCE

Consider a very large randomly mating population and assume a pair of alleles A and A'. If x is the relative frequency of the gene A in the population and s is the selection coefficient of A, the rate of change of the gene frequency due to selection is approximately

$$\delta x = sx(1-x)$$

per generation, when s is small and there is no dominance. If there is random fluctuation in the selection intensity, s and therefore δx are random variables, and a certain irregularity is expected in the process of change in gene frequency from generation to generation. When the rate of change is small, this process may be safely treated as a continuous Markov process.

If $\phi(x,t)dx$ is the probability that the gene frequency lies between x and $x+dx$ in the t-th generation, it can be proved that $\phi(x,t)$ satisfies the partial differential equation,

$$\frac{\partial \phi(x,t)}{\partial t} = \frac{\partial^2}{\partial x^2}\left[\frac{V_{\delta x}}{2}\phi(x,t)\right] - \frac{\partial}{\partial x}\left[M_{\delta x}\phi(x,t)\right], \qquad (1)$$

where $M_{\delta x}$ and $V_{\delta x}$ represent respectively the mean and the variance of δx. This equation which is known by mathematicians as " Kolmogorov's forward differential equation " is usually called " Fokker-Planck equation " by physicists, though this type of equation was already used by LORD RAYLEIGH (cited from FUSHIMI 1941). However, we are indebted principally to SEWALL WRIGHT (1945) for the application of this equation to the problem of population genetics.

A meaning of this equation can easily be understood by noting that the left hand side of this equation represents the rate of change of the relative proba-

bility of any class per generation and this can be decomposed into two parts as represented by two terms in the right; namely the part due to the random fluctuation (first term) and the one due to the directed change (second term).

If the gene A is selectively neutral on the average such that the mean value of its selection coefficient over very long periods is zero,

$$M_{\delta x} = 0 \quad \text{and} \quad V_{\delta x} = V_s x^2 (1-x)^2$$

where V_s is the variance of s. In this case equation (1) is written in the form:

$$\frac{\partial \phi}{\partial t} = \frac{V_s}{2} \frac{\partial^2}{\partial x^2} \{x^2 (1-x)^2 \phi\}. \tag{2}$$

This is a partial differential equation with singularities at the boundaries, so that no arbitrary conditions can be imposed there. But as will be seen in the following operations a continuous stochastic process satisfying the equation (2) is uniquely determined if an initial condition $\phi(x,0)$ is given.

As was demonstrated in the previous report (KIMURA 1952a), if the gene frequency x is transformed into a variate ξ by the relation:

$$\xi = \log\left(\frac{x}{1-x}\right),$$

ξ changes continuously from $-\infty$ to $+\infty$ as x changes from 0 to 1 and the distribution of ξ becomes approximately normal; that is, the process of change of ξ is approximately represented by a Gaussian process.

To solve the equation (2), the same transformation turns out to be very useful: Putting

$$u = \frac{1}{2} e^{\frac{V_s}{8} t} x^{\frac{3}{2}} (1-x)^{\frac{3}{2}} \phi$$

and

$$\xi = \log\left(\frac{x}{1-x}\right),$$

we obtain the heat conduction equation,

$$\frac{\partial u}{\partial t} = \frac{V_s}{2} \frac{\partial^2 u}{\partial \xi^2}. \tag{3}$$

It is already established that this equation has an unique solution which is continuous over $-\infty$ to $+\infty$ when $t \geqq 0$ and which reduces to $u(\xi,0)$ when $t = 0$.

$$u(\xi,t) = \frac{1}{\sqrt{2\pi V_s t}} \int_{-\infty}^{\infty} e^{-\frac{(\xi-\eta)^2}{2V_s t}} u(\eta,0)\, d\eta.$$

Therefore, if the initial distribution of gene frequencies $\phi(x,0)$ is given, the unique solution which satisfies (2) and is continuous between 0 and 1 is

$$\phi(x,t) = \frac{1}{\sqrt{2\pi V_s t}} \frac{e^{-\frac{V_s}{8}t}}{[x(1-x)]^{3/2}} \int_0^1 e^{-\frac{\left[\log \frac{x(1-y)}{(1-x)y}\right]^2}{2V_s t}} \sqrt{y(1-y)}\, \phi(y,0)dy. \quad (4)$$

If the initial condition is not a continuous distribution $\phi(x,0)$, but is a given gene frequency x_0, the relative probability that the gene frequency in the t-th generation will be between x and $x+dx$ is given by the formula:

$$\phi(x,t) = \frac{1}{\sqrt{2\pi V_s t}} \exp\left\{-\frac{V_s}{8}t - \frac{\left[\log \frac{x(1-x_0)}{(1-x)x_0}\right]^2}{2V_s t}\right\} \frac{[x_0(1-x_0)]^{1/2}}{[x(1-x)]^{3/2}}. \quad (5)$$

The process of change of the distribution curve with generations is illustrated in figure 1 assuming that the initial gene frequency in the population is 50%. In this figure the variance of selection coefficient is 0.0483. This is a value which WRIGHT (1948) obtained for the *medionigra* gene in an isolated colony of *Panaxia dominura* (FISHER and FORD 1947), assuming that observed variance of change in gene frequency per year were due wholly to fluctuations in selection. As will be seen in the figure the distribution curve is unimodal before the 28th generation after which it becomes bimodal. In the 100th generation gene frequencies that give maximum probability (corresponding to peaks) are approximately 0.0007 and 0.9993, where the height of the curve (ϕ_{max}) is about 11.37. This is 28.7 times higher than the height at the valley (about 0.397) in the middle part of the distribution. So the distribution curve looks like an U-shaped curve. The more precise form of the terminal part of the distribution curve where the gene frequency is very small is illustrated in figure 2.

With passage of time, the distribution curve becomes nearly U-shaped. The process of change is rather rapid and in the 1000th generation, the peaks of the distribution curve become so high, the gene frequencies corresponding to them become so close to the two termini of the distribution and the valley becomes so deep that it is practically impossible to illustrate the distribution curve in figure 1. More generally, if the initial gene frequency is 50%, the distribution curve is unimodal if the number of generations is less than $4/(3V_s)$ but becomes bimodal if it exceeds this value.

The mean of the distribution is always

$$\int_0^1 x\phi(x,t)dx = x_0, \quad (6)$$

But the variance,

$$V_t = \int_0^1 (x-x_0)^2 \phi(x,t)dx,$$

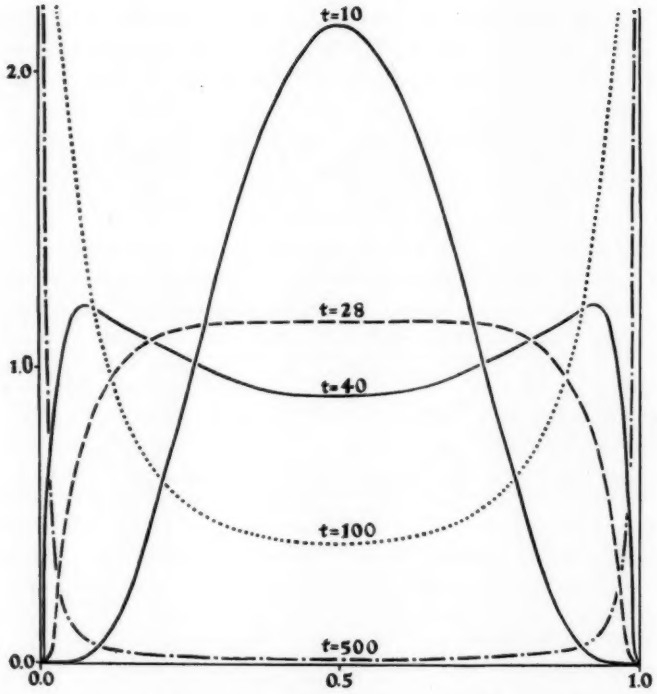

FIGURE 1.—A graph illustrating the process of the change in the distribution of gene frequencies with random fluctuation in the selection intensities. In this illusration it is assumed that the gene is selectively neutral when averaged over a very long period, that there is no dominance, that the initial gene frquncy of the population is 0.5 and that the variance of the selection coefficient is 0.0483. (Abscissa: gene frequency. Ordinate: relative probability.)

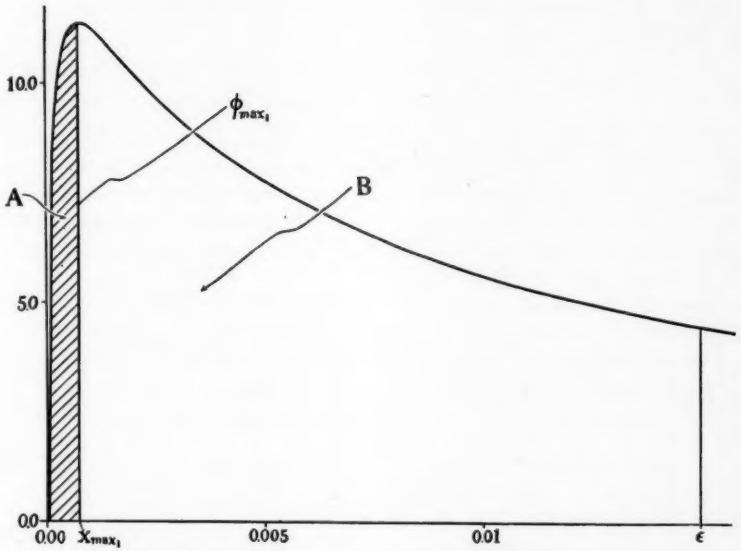

FIGURE 2.—A terminal portion of the distribution curve in the 100th generation where the gene frequency is very low. In this illustration it is assumed that the variance of the selection coefficient is 0.0483 and the initial gene frequency in the population is 0.5. The gene frequency that gives a maximum value in the distribution curve (x_{max_1}) is approximately 0.0007 and corresponding height (ϕ_{max_1}) of the curve is about 11.37. A denotes the probability that the frequency of the gene is smaller than x_{max_1}. This is about 0.007 in this case. ϵ is an arbitrarily chosen gene frequency which is larger than this value. B stands for the probability that the gene frequency in a population is larger than x_{max_1} but smaller than ϵ. If we put ϵ as 0.015, B is approximately 0.098. B becomes ½ at the limit of $t \to \infty$. (Abscissa: gene frequency. Ordinate: relative probability.)

increases in successive generations and for large t it is represented asymptotically by the formula

$$V_t = x_0(1-x_0) - \sqrt{\frac{\pi x_0(1-x_0)}{2V_s t}}\, e^{-\frac{V_s t}{8}} + O\left(\frac{e^{-\frac{V_s t}{8}}}{t\sqrt{t}}\right) \tag{7}$$

Therefore its final rate of approach to the limiting value is very close to $V_\infty/8$ per generation.

CHANGE IN THE TERMINAL PARTS OF THE DISTRIBUTION AND THE PROCESS OF QUASI-FIXATION

As shown above, classes with the highest probability shift toward the terminals indefinitely with time so that the distribution curve appears to be U-shaped. But it is not a true U-shaped curve, since its value at either terminal is always 0. So it will be important to investigate how the distribution curve will continue to change after a sufficient number of generations, with special reference to its terminal parts.

First let us fix our attention to the terminal portion of the distribution where the frequency of the gene A is very low (see figure 2).

The gene frequency x_{max_1} that gives maximum relative probability ϕ_{max_1} is asymptotically

$$x_{max_1} \sim \frac{x_0}{1-x_0}\, e^{-\frac{3}{2}V_s t} \to 0 \quad (t \to \infty). \tag{8}$$

While the corresponding relative probability is

$$\phi_{max_1} \sim \frac{1}{\sqrt{2\pi V_s t}} \frac{(1-x_0)^2}{x_0}\, e^{V_s t} \to \infty \quad (t \to \infty). \tag{9}$$

The gene frequency that gives the maximum value in the distribution curve will approach indefinitely to one terminal point (0), elevating indefinitely the corresponding height of the distribution curve.

Let A be the probability that the gene frequency in the population is lower than x_{max_1}. To calculate this, we will start from a more general relation: The probability that the gene frequency in the population falls between two assigned values a and b is

$$\Pr\{a < x < b\} = \int_a^b \phi(x,t)dx$$

$$= \frac{1-x_0}{\sqrt{2\pi}} \int_{\alpha(a)}^{\alpha(b)} e^{-\frac{1}{2}\lambda^2}\, d\lambda + \frac{x_0}{\sqrt{2\pi}} \int_{\beta(a)}^{\beta(b)} e^{-\frac{1}{2}\lambda^2}\, d\lambda, \tag{10}$$

where

$$\alpha(\lambda) = \frac{\log\left(\frac{\lambda}{1-\lambda}\right) - \log\left(\frac{x_0}{1-x_0}\right)}{\sqrt{V_s t}} + \tfrac{1}{2}\sqrt{V_s t}$$

and

$$\beta(\lambda) = \frac{\log\left(\frac{\lambda}{1-\lambda}\right) - \log\left(\frac{x_0}{1-x_0}\right)}{\sqrt{V_s t}} - \tfrac{1}{2}\sqrt{V_s t}.$$

Using this relation it can be easily shown that

$$A = \Pr\{0 < x < x_{max_1}\}$$

$$\sim \frac{1-x_0}{\sqrt{2\pi}} \int_{\sqrt{V_s t}}^{\infty} e^{-\frac{1}{2}\lambda^2} d\lambda + \frac{x_0}{\sqrt{2\pi}} \int_{2\sqrt{V_s t}}^{\infty} e^{-\frac{1}{2}\lambda^2} d\lambda$$

$$= \frac{1-x_0}{\sqrt{2\pi V_s t}} e^{-\frac{1}{2}V_s t} + O\left(\frac{e^{-2V_s t}}{\sqrt{V_s t}}\right) \to 0. \tag{11}$$

That is, this probability vanishes at the limit; $t \to \infty$.

On the other hand, Let B stand for the probability that the gene frequency in the population is larger than x_{max_1} but smaller than ϵ, where ϵ is an arbitrarily chosen gene frequency larger than x_{max_1}. Using the relation (10),

$$B = \int_{x_{max_1}}^{\epsilon} \phi(x,t)dx \sim \frac{1-x_0}{\sqrt{2\pi}} \int_{-\sqrt{V_s t}}^{\frac{\log\epsilon - \log\left(\frac{x_0}{1-x_0}\right)}{\sqrt{V_s t}} + \frac{1}{2}\sqrt{V_s t}} e^{-\frac{1}{2}\lambda^2} d\lambda$$

$$+ \frac{x_0}{\sqrt{2\pi}} \int_{-2\sqrt{V_s t}}^{\frac{\log\epsilon - \log\left(\frac{x_0}{1-x_0}\right)}{\sqrt{V_s t}} - \frac{1}{2}\sqrt{V_s t}} e^{-\frac{1}{2}\lambda^2} d\lambda.$$

Therefore, for any ϵ, however small, B can be brought arbitrarily close to $1 - x_0$ by taking t sufficiently large such that

$$V_s t \gg -\log \epsilon.$$

This may be made more clear by the following relation:

$$B = \Pr\{x_{max_1} < x < \epsilon\}$$

$$\sim \frac{1-x_0}{\sqrt{2\pi}} \int_{-\sqrt{V_s t}}^{\frac{1}{2}\sqrt{V_s t}} e^{-\frac{1}{2}\lambda^2} d\lambda + \frac{x_0}{\sqrt{2\pi}} \int_{-2\sqrt{V_s t}}^{-\frac{1}{2}\sqrt{V_s t}} e^{-\frac{1}{2}\lambda^2} d\lambda$$

$$= (1 - x_0) - \frac{2(1 - 2x_0)}{\sqrt{2\pi V_s t}} e^{-\frac{V_s t}{8}} + O\left(\frac{e^{-\frac{V_s t}{2}}}{\sqrt{V_s t}}\right) \to 1 - x_0 \ (t \to \infty). \quad (12)$$

This shows that after a sufficient number of generations B approaches to $1 - x_0$ with the rate of $V_s/8$ per generation. Figure 2 illustrates the terminal portion of the distribution curve when $V_s = 0.0483$ and $x_0 = 0.5$.

Similar relations hold for the other terminal portion of the distribution where the frequency of the gene is very close to 1: It x_{max_2} stands for the gene frequency giving the maximum value in the distribution curve and ϕ_{max_2} stands for the corresponding relative probability,

$$x_{max_2} \sim 1 - \frac{1 - x_0}{x_0} e^{-\frac{3}{2} V_s t} \to 1 \quad (13)$$

and

$$\phi_{max_2} \sim \frac{1}{\sqrt{2\pi V_s t}} \frac{x_0^2}{1 - x_0} e^{V_s t} \to \infty \quad (14)$$

as $t \to \infty$. The probability A′ that the frequency of the gene exceeds x_{max_2} vanishes as t approaches infinity;

$$A' = Pr\{x_{max_2} < x < 1\} \sim O\left(\frac{e^{-\frac{1}{2} V_s t}}{\sqrt{V_s t}}\right) \to 0. \quad (15)$$

On the other hand, even if $\epsilon' (> 0)$ is taken however small, the probability B′ that the gene frequency of the population will fall between $1 - \epsilon'$ and x_{max_2} approaches to x_0 with the rate of $V_s/8$ per generation at the limit of $t \to \infty$;

$$B' = Pr\{1 - \epsilon' < x < x_{max_2}\} \sim x_0 + O\left(\frac{e^{-\frac{V_s}{8} t}}{\sqrt{V_s t}}\right). \quad (16)$$

The gene frequency giving the minimum of this pseudo-U-shaped distribution curve (x_{min}) approaches ½ at $t \to \infty$ even if the initial gene frequency is not 50%:

$$x_{min} \sim \frac{1}{2} - \frac{\log \frac{x_0}{1 - x_0}}{\frac{3}{2} V_s t - 4}. \quad (17)$$

The corresponding relative probability ϕ_{min} vanishes at the limit:

$$\phi_{min} \sim \sqrt{\frac{x_0(1-x_0)}{2\pi V_s t}} \left\{ \frac{1}{4} - \left(\frac{\log \frac{x_0}{1-x_0}}{\frac{3}{2}V_s t - 4} \right)^2 \right\}^{3/2} \exp\left\{ -\frac{V_s t}{8} - \frac{9}{8}\left(\frac{\log \frac{x_0}{1-x_0}}{\frac{3}{2}V_s t - 4} \right)^2 V_s t \right\} \to 0. \quad (18)$$

That is, the valley in the distribution curve deepens until the bottom reaches the abscissa.

As will be seen from the relation;

$$\lim_{\epsilon \to 0+} \int_{\epsilon}^{1-\epsilon} \phi(x,t) = 1, \quad (19)$$

the random fluctuation of selection intensities by itself cannot lead to the complete fixation or loss, in the strict sense, of the gene contrary to the case of random drift due to small population number. But as has been shown through (8) – (18), there is a strong tendency that the gene frequency will move toward either terminus with increasing time. In other words, after a sufficient number of generations almost all populations will be in such a situation that the gene is either almost fixed in the population or almost lost from it. To distinguish this from the fixation or loss in the case of small effective population number, the terms " quasi-fixation " and " quasi-loss " are proposed. As will be seen from (12) and (16), their rate can be taken as $V_s/8$.

In the long run, this process of quasi-fixation or -loss will be checked by the opposing mutation pressure.

In this state of statistical equilibrium, if the mutation rates of the gene A to and from its allele A' are u and v respectively, the frequency distribution of A in the population is given by the formula:

$$\phi(x) = C \frac{e^{-\frac{2}{V_s}\left(\frac{v}{x} + \frac{u}{1-x}\right)}}{x^2(1-x)^2} \left(\frac{x}{1-x} \right)^{-2\left(\frac{u-v}{V_s}\right)}, \quad (20)$$

where C is a constant chosen such that $\int_0^1 \phi(x)dx = 1$. WRIGHT (1948) derived essentially the same formula assuming migration (p. 292).

COMPARISON WITH THE PROCESS OF RANDOM DRIFT DUE TO SMALL POPULATION NUMBER

In a finite population, owing to random sampling of gametes in reproduction, there occurs random fluctuation of the gene frequency from generation to generation. This process, as is well known, will finally lead to the complete fixation or loss of the gene if such factors as mutation, migration and selection are absent.

If N is the effective number of reproducing individuals in the population and p is the initial gene frequency the nth moment of distribution about zero in the t-th generation is given by the following formula if the order of t is not smaller than N (cf. KIMURA 1952b):

$$\mu_n'(t) = p - 3pq\frac{n-1}{n+1}(1-\lambda_1)^t - 5pq(p-q)\frac{(n-2)(n-1)}{(n+1)(n+2)}(1-\lambda_2)^t$$
$$- 7pq(-5pq+1)\frac{(n-3)(n-2)(n-1)}{(n+1)(n+2)(n+3)}(1-\lambda_3)^t$$
$$- 9pq(14pq^2-7pq+p-q)\frac{(n-4)(n-3)(n-2)(n-1)}{(n+1)(n+2)(n+3)(n+4)}(1-\lambda_4)^t$$
$$+ O\{(1-\lambda_5)^t\}, \quad (21)$$

where $q = 1-p$. From this we can derive the probability that the gene will have become fixed in the population by the t-th generation:

$$f_t(1) = p - 3pq(1-\lambda_1)^t - 5pq(p-q)(1-\lambda_2)^t - 7pq(-5pq+1)(1-\lambda_3)^t$$
$$- 9pq(14pq^2-7pq+p-q)(1-\lambda_4)^t + O\{(1-\lambda_5)^t\} \quad (22)$$

The corresponding probability of complete loss is:

$$f_t(0) = q - 3pq(1-\lambda_1)^t + 5pq(p-q)(1-\lambda_2)^t - 7pq(-5pq+1)(1-\lambda_3)^t$$
$$+ 9pq(14pq^2-7pq+p-q)(1-\lambda_4)^t + O\{(1-\lambda_5)^t\}(t\to\infty). \quad (23)$$

In these formulae

$$\lambda_1 = \frac{1}{2N}, \lambda_2 = \frac{3}{2N}, \lambda_3 = \frac{6}{2N}, \lambda_4 = \frac{10}{2N}, \lambda_5 = \frac{15}{2N}, \ldots.$$

In general λ's are given by the formula;

$$\lambda_i = \frac{i(i+1)}{4N} \quad (i = 1, 2, \ldots).$$

The frequency of the gene in this case may take any one of a series of discontinuous values:

$$0, \frac{1}{2N}, \frac{2}{2N}, \ldots, 1-\frac{1}{2N}, 1.$$

Usually, however, the number of reproducing individuals (N) in a population is so large that practically the gene frequency (x) can be treated as a continuous variable with good approximation. Variance of the rate of change in gene frequency due to the random sampling of gametes is

$$V_{\delta x} = \frac{x(1-x)}{2N}.$$

Therefore if $\phi(x,t)$ is the relative probability that the frequency of the gene in the population will take any value between x and $x+dx(0<x<1)$ in the t-th generation, $\phi(x,t)$ satisfies the following partial differential equation:

$$\frac{\partial \phi}{\partial t} = \frac{1}{4N}\frac{\partial^2}{\partial x^2}\{x(1-x)\phi\}, \tag{24}$$

which is easily derivable from equation (1). To solve this, if we put

$$\phi \propto X_i(x)e^{-\lambda_i t} \qquad (i = 1, 2, 3, \ldots),$$

we obtain the ordinary differential equation;

$$x(1-x)\frac{d^2X_i}{dx^2} + (2-4x)\frac{dX_i}{dx} - (2-4N\lambda_i)X_i = 0,$$

where λ_i corresponds to the eigen value of equation (24). Noting that $\lambda_i = i(i+1)/4N$, this becomes

$$x(1-x)\frac{d^2X_i}{dx^2} + (2-4x)\frac{dX_i}{dx} - (1-i)(i+2)X_i = 0. \tag{25}$$

This type of equation is known as Gauss's differential equation and (25) is satisfied by the following hypergeometric series:

$$\begin{aligned} X_i &= F(1-i,\ i+2,\ 2,\ x) \\ &= 1 + \frac{(1-i)(i+2)}{1\cdot 2}x + \frac{(1-i)(2-i)\cdot(i+2)(i+3)}{1\cdot 2\cdot 2\cdot 3}x^2 \\ &\quad + \frac{(1-i)(2-i)(3-i)\cdot(i+2)(i+3)(i+4)}{1\cdot 2\cdot 3\cdot 2\cdot 3\cdot 4}x^3 \\ &\quad + \frac{(1-i)(2-i)(3-i)(4-i)\cdot(i+2)(i+3)(i+4)(i+5)}{1\cdot 2\cdot 3\cdot 4\cdot 2\cdot 3\cdot 4\cdot 5}x^4 \\ &\quad + \ldots \ (i = 1, 2, 3, \ldots) \end{aligned}$$

Therefore the asymptotic solution of (24) for large t is;

$$\begin{aligned} \phi(x,t) = {} & C_1e^{-\lambda_1 t} + C_2(1-2x)e^{-\lambda_2 t} + C_3(1-5x+5x^2)e^{-\lambda_3 t} \\ & + C_4(1-9x+21x^2-14x^3)e^{-\lambda_4 t} + O(e^{-\lambda_5 t}). \end{aligned} \tag{26}$$

To determine the constants C_1, C_2, C_3, . . . we can use the relation that the n-th moment of distribution obtained by this formula,

$$\int_0^1 x^n \phi(x,t)\,dx,$$

must be equal to

$$\mu'_n(t) - 1^n \cdot f_t(1),$$

since the homallelic classes are excluded from the distribution curve to be given by (24). Thus we obtain the following values:

$$C_1 = 6\,pq, C_2 = -30\,pq(p-q), C_3 = 84\,pq(-5\,pq+1),$$
$$C_4 = -180\,pq(14\,pq^2 - 7\,pq + p - q).$$

How the distribution curve represented by (26) changes with generations is illustrated in figure 3, assuming that the initial gene frequency p is 0.1. As may be seen from this figure, the curve becomes gradually flat until finally every heterallelic class has equal probability and falls with the rate of 1/2N per generation. In this final stage, the fixation of the gene proceeds at the same rate, the correct value of which was first obtained by WRIGHT to be 1/2N (see WRIGHT 1931) by using a different method of calculation. This rate is usually known as the rate of fixation due to random sampling of gametes.

The probability Ω_t that the alleles A and A′ coexist in the population in the t-th generation can be obtained from (26):

$$\Omega_t = \int_0^1 \phi(x,t)\,dx$$

$$= 6\,pq\, e^{-\frac{1}{2N}t} + 14\,pq\,(-5\,pq+1)\, e^{-\frac{6}{2N}t} + O\left(e^{-\frac{15}{2N}t}\right) (t \to \infty). \quad (27)$$

Contrary to what was shown in (19) either complete fixation or loss of alleles is expected in this case and Ω_t vanishes at the limit of $t \to \infty$.

Variance of the distribution in the t-th generation is from (22), (23) and (26),

$$V_t = pq - pq\, e^{-\frac{1}{2N}t}, \quad (28)$$

namely the variance approaches its limiting value pq at the rate 1/2N per generation.

As has been demonstrated above, the process of change due to random fluctuation of selection intensity is quite different from that due to the random sampling of gametes. Therefore comparison of their effects must be made from various angles as WRIGHT (1948) did in analyzing the data of *medionigra* gene in Panaxia.

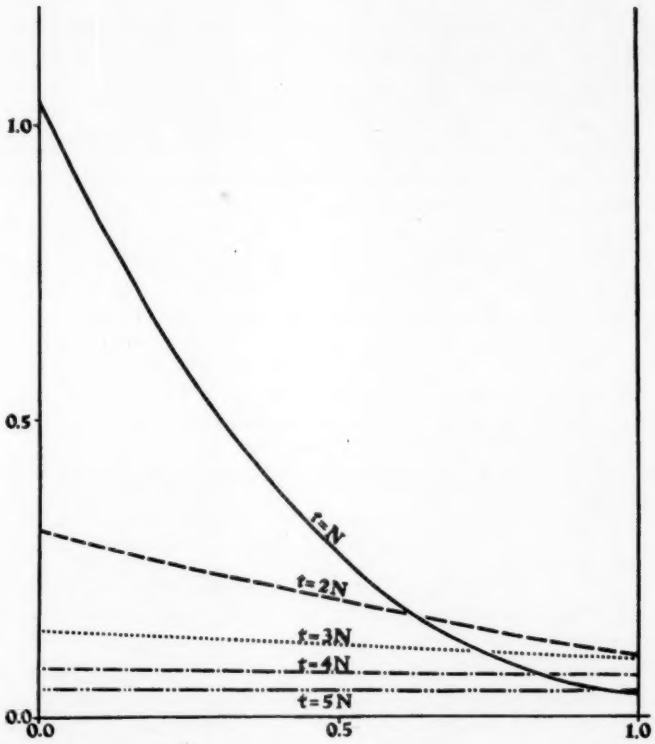

FIGURE 3.—Graphs showing the process of change of the frequency distribution curve due to the random drift in small populations. In this illustration it is assumed that the initial gene frequency in the population is 0.1. It will be seen that the distribution curve becomes more and more flat as the number of generations increases. (Abscissa: gene frequency in the population. Ordinate: relative probability. t: time in generation. N: effective population size.)

Thus, if we consider the process of change which will be realized after a sufficient number of generations, the rate of quasi-fixation, $V_s/8$, may be compared with the rate of fixation due to random sampling, $1/2N$, for the same purpose. Suppose V_s is known to be 0.0483 as in figure 1, the equivalent N is calculated to be about 83 by using a equivalence relation;

$$\frac{1}{2N} \doteq \frac{V_s}{8},$$

though the applicability of this formula is rather restricted.

So far we have treated the two factors separately. But in nature not only these two factors but also systematic factors may work concurrently. The present writer (1951) reported briefly on the distribution of gene frequencies for such case. The more precise account will appear elsewhere, but the main conclusion derived from the analysis of the distribution curve is not difficult to present here: The effect produced by the random fluctuation in natural selection is relatively unimportant for small populations. But in large populations it has a remarkable effect in such ways that in the case of no dominance the distribution curve is modified markedly in the parts where the frequency of either allele is low and in the case of complete dominance in a part where the frequency of the recessive gene lies inside a certain range of higher frequencies. Also the product NV_s is an important quantity. To estimate not only the distribution of population size (N) in nature but also the variability (V_s) of selection intensity for important loci may be an important task left for the future experiments.

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SUMMARY

When there are random fluctuations in selection intensity, the process of change in gene frequency in a population is represented by a stochastic process. In this paper an analysis of this process is presented for a gene lacking dominance and selectively neutral on the average. Especially interesting is the process of change that can be observed in the terminal portions of the distribution curve. Contrary to the case of random drift in small populations, if the population is very large, complete fixation or loss of an allele, in the strict sense, will not be realized. But there exists a strong tendency toward the state of almost fixation or almost loss. That is, if we allow a sufficient number of generations a situation will almost surely be realized in which the allele is either almost fixed in the population or almost lost from it. To distinguish this

from the fixation or loss in the case of random drift in small populations, terms "quasi-fixation" and "quasi-loss" were proposed. Their rate per generation can be taken as $V_s/8$, where V_s is the variance of the selection coefficient. Comparison of this process with that of random drift in small populations is another important subject in the present paper. In spite of many studies on the process of the drift very little is known about the process of the change before the fixation and loss of an allele proceeds at the constant rate of $1/2N$. In this paper an asymptotic solution for this process is presented for the first time.

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THE QUANTITATIVE ESTIMATION OF RADIATION INDUCED MUTATIONS TO STREPTOMYCIN RESISTANCE IN ESCHERICHIA COLI

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IN our studies of the genetic effects of radiation and radioactive isotopes, it was necessary to develop a quantitative measure of mutation rate which would remain reliable under the peculiar conditions of the requisite experiments (RUBIN 1948, 1949, 1950a; RUBIN and STEINGLASS 1949, 1950). This paper describes some observations made during the evolution of such a method.

Quantitation of the change in bacteria from streptomycin sensitivity to resistance was taken for detailed study for several reasons. Streptomycin (sulfate) is very stable under the most extreme conditions of our experiments and has the very desirable characteristic of rapidly destroying the background of sensitive organisms. Even rare mutants, therefore, can be readily demonstrated in very large populations of sensitive cells (DEMEREC 1951; RUBIN et al. 1951). Resistance to this drug (at levels exceeding 25 μg per ml) seems to be controlled by a single genetic locus (NEWCOMBE and NYHOLM 1950) but several presumably allelic changes may be recognized (DEMEREC 1951). Thus completely resistant mutants may be obtained in the course of a single selection from a concentration of the drug which is immediately lethal to the large mass of sensitive cells. Resistant clones can then be qualitatively characterized and each mutant can be shown to have a stable type of resistance to the drug, changing only by another mutation and selection process, in a manner clearly described by BERTANI (1951).

METHODS AND EXPERIMENTATION

The culture used in this study was *Escherichia coli B/r*, obtained from the Carnegie Institution at Cold Spring Harbor. The streptomycin was all streptomycin sulfate of one single batch purchased from the Heyden Chemical Company. Irradiation was achieved by means of a deep therapy medical X-ray unit in a manner previously described (RUBIN 1950). In the present experiments all X-radiation was done at a standard distance and setting (160 Kv and 10 ma) to provide 5000 roentgens per hour uniformly. Irradiation was always carried out at 37°C under conditions which permitted growth of the organisms (RUBIN and STEINGLASS 1950).

When aliquots of irradiated cultures are plated on agar containing streptomycin, surviving colonies (mutants) are counted with a stereoscopic microscope after at least four days of incubation at 37°C. Further incubation causes no significant increase in the numbers of mutant colonies. Controls (back-

ground mutations) of unirradiated cultures were always determined from aliquots of the inoculum used for the irradiations.

Whole culture counts on controls and irradiated cultures were done in triplicate. Three separate dilution series were made and at least three replicate plates (Petri dishes of 100 by 20 mm) of each dilution level were counted. At least nine plates were thus employed for each determination of total viable count.

All counts and mutant (streptomycin) plates were done by the multiple agar layer technique (LEDERBERG and TATUM 1946) in which the layer containing bacteria is sandwiched between two sterile layers of the same kind of agar. The counts were made on Difco Nutrient Agar, and the mutant plates on the various media to be described below. The layer plate technique was found to give greater quantitative reproducibility and to make microscopic counting easier. When contaminants appeared, they were usually confined to the surface of the agar and could be readily distinguished from the colonies of interest, which were embedded at a uniform interior level.

Usually few streptomycin-resistant cells are found in cultures plated immediately after irradiation. If a period of growth is permitted to intervene before plating, the proportion of mutants increases sharply. The characteristic pattern of increase and decline in the frequency of mutants may be seen in figure 1. A quantitation of this pattern was attempted by assaying a series of rapid subcultures in which 2 ml of a fully grown culture was added to 6 ml of sterile medium (two generations of growth permitted) and repeated as soon as growth was complete. The proportion of mutants in each subculture was determined by plating about 4 ml in from five to ten agar layer plates containing 250 μg of streptomycin per ml. The rest of the culture was used to inoculate the subsequent subculture and for the determination of total viable count.

Since none of the mutant clones could grow faster than the non-mutants (DEMEREC 1951), the increase is clearly the result of delays in the phenotypic expression of resistance. But the sharpness of the peak and the rapidity of decline makes it evident that the loss of the more poorly surviving mutants has started even while the curve is rising. This was corroborated by an examination of the characteristics of each of the mutants found in several subculture series, which showed a disproportionately rapid decline in mutants requiring streptomycin for growth. That such a dynamic process is difficult to quantitate is shown by the examples plotted in figure 1. Although replicate irradiations gave strikingly different curves, a twofold increase in radiation dose failed to cause a proportionate increase in measurable genetic effect. In spite of careful study, no variation of the subculture technique was found capable of providing dependable quantitative data.

NEWCOMBE's (1952) adaptation of phage techniques (DEMEREC 1946) provided a key to the development of methods more suitable for the quantitation of the genetics of streptomycin resistance. As modified in the present experiments, this procedure consists of sandwiching irradiated bacteria embedded in a center agar layer between two sterile layers of the same (nutrient) agar.

After several hours of incubation at 37°C, a fourth agar layer containing streptomycin is added. The plates are then refrigerated to permit diffusion of the drug, and finally the plates are again incubated at 37°C to permit the full development of the mutant colonies.

The period of preincubation at 37°C before adding streptomycin is required to provide completion of the delayed phenotypic expression. NEWCOMBE noted that if this period was longer than six hours, the count of mutants decreased sharply. Our studies of this preincubation period showed that the time required

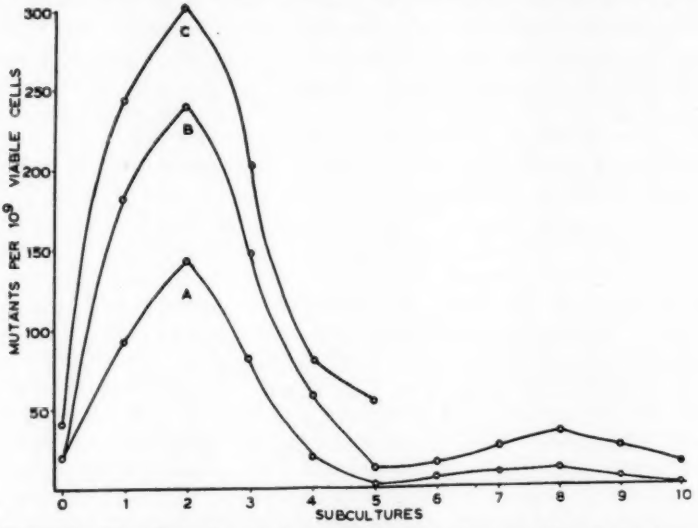

FIGURE 1.—The effect of successive subculturing on the proportion of streptomycin-resistant organisms found in irradiated cultures. The "zero point" is determined by plating aliquots of the culture immediately after irradiation. This same cuture (2 ml) is then used to seed 6 ml of sterile nutrient broth. As soon as growth in this "first subculture" is complete, samples are taken for plating and subsequent subculture. Each point is the average number of colonies appearing on 10 plates containing 250 μg of streptomycin per ml of agar. Curves A and B are from replicate cultures irradiated simultaneously for three hours at 5000 roentgens per hour. Curve C is from a culture irradiated for six hours under the same conditions.

for the maximum yield of mutants varied with the size of the aliquot of irradiated culture on the plate. When more than about 4×10^8 viable irradiated cells were plated, the changes resulting from the variation of preincubation time were similar to those seen in the subculturing technique. No stable plateau value was ever achieved. But when fewer organisms were added, the maximum number of mutants was regularly expressed after about three hours, and there was no appreciable variation during the next seven hours (figure 2).

That the failure to obtain such a plateau is due only to an excess of viable cells was shown by adding washed unirradiated cells to small aliquots of

irradiated cultures. While living cells inhibited the formation of a plateau and diminished the mutant count, equal numbers of heat-killed cells had no such effect. The addition of various amounts of irradiated culture medium (after removal of the cells by centrifugation) also had no effect on either the number of mutants or the pattern of expression in this layer plate technique.

It may be noted in figure 2 that the maximum number of mutants detectable does not increase proportionately with the increase in culture volume. A similar observation was made by BRYSON (1951) who actually found a continuous decrease in detectable mutants as the number of irradiated cells exceeded 10^8 per plate. To find the lower limit of the inhibiting effect of popula-

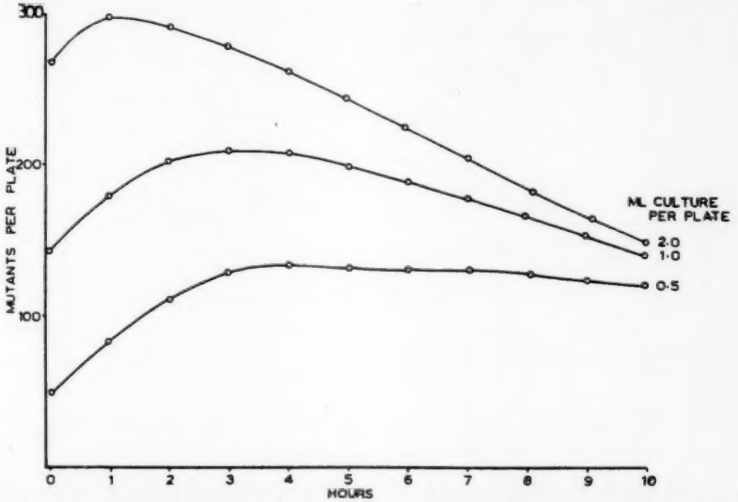

FIGURE 2.—The effect of preincubation time (before adding streptomycin) on the number of mutant colonies detectable. The culture contained 8.2×10^8 viable cells per ml after irradiation for 4.5 hours at the rate of 5000 roentgens per hour. The streptomycin added in the fourth layer (after preincubation) was calculated to provide 100 μg per ml after diffusion. Each point is the average of 18 replicate plates.

tion pressure we made comparisons of small culture concentrations. Complete proportionality was not achieved even when only 10^7 cells were plated. Smaller aliquots are difficult to test because the small number of mutants per plate introduces a large statistical error. Within practical limits, therefore, the smaller the population tested, the larger the proportion of mutants.

From figure 3 it is evident that when there are fewer than 10^9 cells per plate the relationship between the volume of culture plated and the number of mutants detected forms a simple linear function which may be extrapolated to zero population. The comparison of different irradiations, using this extrapolated point, shows it to be the most stable value to be found. Since the slope of the line is not constant for different irradiations, it is necessary each time

to obtain enough points in the linear region. When this is done, the agreement between experiments is as good as careful plate counting permits.

The reliability of such data depends on the size of the mutant count and the number of replicate plates. Large numbers of replicates could be prepared rapidly by pipetting the irradiated culture into a large flask of melted agar, mixing, and then dispensing the agar into several Petri dishes. For reasons of convenience in our laboratory, we usually used 500-ml flasks containing 300 ml of agar, and dispensed this into nine Petri dishes. It was found that the total mutant count per flask depends on the concentration of viable cells in the middle agar layer and not upon the volume of seeded agar per plate.

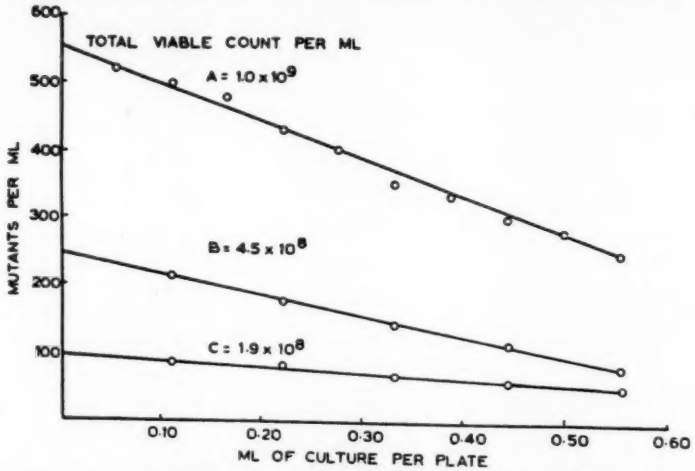

FIGURE 3.—Mutant frequency as a function of the volume of irradiated culture per plate. Three cultures were irradiated equally (5000 roentgens per hour for three hours) but at different times. By extrapolating to the point of no population effect (y axis) the mutant frequency per 10^9 viable cells is 550 for A, 535 for B, and 525 for C. Each point represents the average of 36 replicate plates.

In practice it was convenient to pour the contents of a flask freely (without measuring) into nine plates and then to count the nine as one unit. At least two such units of nine plates were counted for each point, and more if the mutation rate was low. The values for figure 3 were obtained in this manner. It may be noted that the average volume per plate plotted is 1 ml divided by 9; 2 ml divided by 9, etc. In this way a single pipetting of a large volume obviates the necessity for many pipettings of small volumes. The variable in figure 3 is the number of organisms per unit volume of seeded agar rather than per plate, but since these relationships were kept constant, it is equivalent to organisms per plate. Therefore, if the culture is pipetted directly onto the plate, the middle agar layer must also be carefully measured.

The preincubation time was standardized at 5 hours, a period well within

the plateau region and long before there is any tendency for the mutant count to decline. The refrigeration period (after the addition of streptomycin) usually lasted about 18 hours (overnight). No significant change in mutant count occurred by continued refrigeration as long as 96 hours. The period of preincubation in these experiments was ended very sharply by cooling the plates on a refrigerated table surface (a " Dole Plate " such as is used to keep food cold in cafeterias). The fourth layer containing streptomycin was then added to the cold Petri dishes, and the plates immediately refrigerated.

If all the layers were to be poured freely (without measurement) it was important to know the effect of streptomycin, since there was bound to be

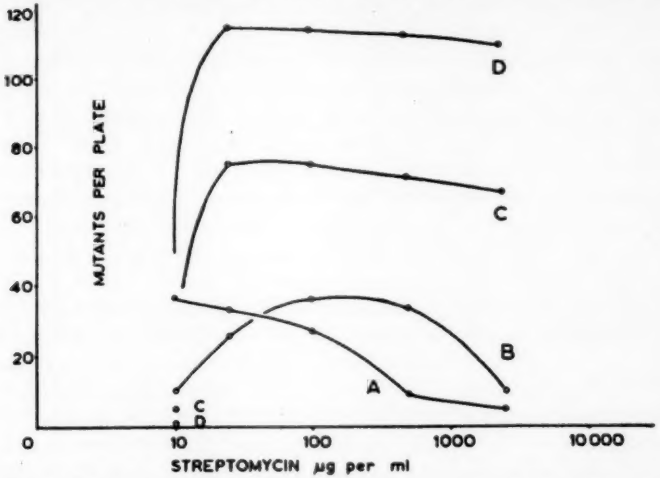

FIGURE 4.—The effect of varying the streptomycin concentration in different media on the number of mutants found in an irradiated culture. Equal aliquots of the same culture (2.1×10^8 viable cells per ml) were plated on: *A*—Streptomycin Assay (S.A.) Agar pH 8 in all four layers. *B*—Nutrient Agar (N.A.) pH 6.8, in all four layers. *C*—N.A. in the first three layers and B.H.I. in the fourth. *D*—S. A. in the first three layers and Brain-Heart Infusion (B.H.I.) in the fourth. Each point is the average of nine plates.

some variation in concentration. Figure 4 illustrates the effect of wide variations in streptomycin concentration on the frequency of mutants from equal aliquots from the same culture. In these experiments the volume of all the layers was carefully measured, and the concentration of streptomycin adjusted to give exactly the desired level. The periods of preincubation, refrigeration, and final incubation were carried out in the standardized 5, 18 and 96 hours respectively.

From such results it was clear that under readily attainable conditions there was little difference in the selective effect of streptomycin from 25 to 2500 μg per ml. Therefore, the method was further standardized to use agar containing 500 μg per ml in the fourth layer, which gives a final concentra-

tion of about 100 μg per ml throughout the agar, after diffusion. An error in either direction of over 100 percent would make no difference in the number of mutants detectable. Numerous experiments were carried out to compare the data from plates in which all layers were poured freely and from those in which everything was carefully measured. Variation in count among replicate plates was about equal for both methods, and there was no significant difference in mutant frequency. It was thus possible to confidently avoid the multiple pipetting of small volumes and the measuring of melted agar just a few degrees above the solidification point.

It can be seen in figure 4 that the composition of the medium influences the response to streptomycin. When either Difco Streptomycin Assay Agar or Difco Nutrient Agar is used in all four layers the kind of response to variations in streptomycin concentration is less uniform than when Difco Brain Heart Infusion Agar is used for the fourth layer, and the difference in the number of mutants obtainable is quite striking. In previous experiments it had been noted that when mutants were cultured for study, more clones survived in Brain Heart Infusion Broth than in any other medium tried. In Nutrient Broth, for instance, fewer than 60 percent of the mutant clones could survive even if they had first appeared on Streptomycin Agar containing the same nutritive ingredients. In Brain Heart Infusion virtually all clones so detected did survive.

It was not feasible to use Brain Heart Infusion Agar all through a " layer plate " because of its glucose content. In the preincubation period the glucose would be fermented to produce a gassy plate, worthless in the layering technique. It was possible to use this medium in the fourth layer (which also contains the streptomycin) because the number of colonies that grew out in streptomycin is relatively small and the gas formation could then be completely inhibited by using an alkaline medium in the first three layers. By comparing different concentrations of Brain Heart Agar it was shown that its beneficial effect on the survival capacity of mutants could be achieved simply by the metabolites diffusing from a single 10-ml layer. Multiplying the concentration of the Brain Heart Infusion or increasing the volume of the fourth layer had no additional effect. Several other supplements, including nucleic acid mixtures, yeast extract, etc., were tested and found not as effective as the commercial Brain-Heart mixture.

In figure 4 it can be seen that the composition of the lower three layers also affects the final yield of mutants. It appeared advisable to use the " Assay " Agar (pH 8) in the first three layers, both to give a higher yield of mutants and also to suppress the formation of gas from the fermentation of the diffusing glucose. Other experiments (RUBIN et al. 1951) also indicated the advisability of using a medium of about pH 8 for the selection of streptomycin-resistant mutants. At more acid levels, slight changes in pH or streptomycin concentration can seriously change the effect of the drug on sensitive cells. But at a clearly alkaline level, the antibacterial and lytic effect is uniformly rapid and complete.

Another variable to be considered is the temperature of the agar in which the irradiated organisms are dispersed. It was noted that if this temperature was even slightly above 45°C, there was a serious loss of mutants. Since this temperature does not seem to greatly affect t e non-mutant cells, it has been suggested that there may be a radiation reacti ating effect at higher temperatures (ANDERSON 1951). In any case, it was necessary to be certain that the agar was below 45°C. This was done by storing the agar for several hours in water baths or incubators held at about 45°C. The actual temperatures of pouring of the middle layers was 40–43°C. Rapid cooling was assured by spreading the plates on refrigerated table tops.

DISCUSSION

The extensive interest in the genetic effect of radiations, isotopes, carcinogens, etc., has stimulated the study of quantitative mutation rates in microorganisms. The desirability of using bacteria and the genetic validity of these studies have been discussed extensively but the limitations of the available techniques are not sufficiently appreciated (LEDERBERG 1948).

Until very recently the only available quantitative mutation technique in bacteria was the test for resistance to phage (DEMEREC 1946). But this involved several rather tricky techniques and remained open to a number of theoretical reservations. The long period required for complete phenotypic expression and the large proportion of zero point mutants led even DEMEREC to seek some more suitable techniques (DEMEREC 1951; DEMEREC and CAHN 1953).

Recently, quantitative mutation studies in bacteria have explored the use of genetic reversions—the loss of dependence on some metabolite or drug. The careful studies of BERTANI (1951) show the characteristic difficulties of such techniques. It is necessary to allow for the full phenotypic expression of the delayed mutations after mutagenic treatment. This would require a period of growth, and the increase of the non-mutant cells which are thereafter *not* eliminated. BERTANI solved this problem by pre-growing his cells before irradiation in a limited concentration of the substance on which they are dependent (streptomycin). He found that as he increased the concentration of streptomycin in this early growth period, he also increased the detectable number of mutants. He could not, however, reach a maximum since this would provide enough streptomycin to cause excessive growth of the non-mutants and inhibition of the mutants. This, then, is the basic difficulty of the reversion technique, there is always some background growth of the non-mutants which is hard to control while insuring complete, or even a predictable level of phenotypic expression of the mutants. DEMEREC and CAHN (1953) have devised a similar but even more complex system requiring a delicate balance between the small amount of added nutrient and the number of treated organisms plated.

The "streptomycin layer technique," first used by NEWCOMBE and then studied by DEMEREC, permits the careful control of the factors influencing

complete expression of the mutants. NEWCOMBE did not consider the effect of population size on the pattern of genetic expression, and while DEMEREC did recognize this problem he could not solve it by means of the spraying technique which he modified from his phage methods. The present resolution of this problem shows that there is no really simple solution. Even though the pattern of genetic expression can be carefully standardized in this method, there is no way of getting around the effects of population pressure. A tempting thought is that if a higher mutation rate were available, this effect of population would be lost. But even this is probably illusory. In the course of ordinary plate counting, it is common experience to note that as the aliquot of culture on the plate increases, the apparent total viable count decreases, especially if the colonies per plate exceed 1000. This limitation of the total count on a single Petri dish was nicely described by BEALE (1948) who noted how it complicated the phage resistance technique.

If one can resign himself to the inevitable statistical problems of genetic techniques, the procedures described for obtaining a slope rather than the usual single point are really not too laborious. Actually it was frequently more trouble to obtain reliable total plate counts than the points needed to plot the mutation rate.

The statistics of this technique were improved by the use of the Brain-Heart Infusion supplement—many more mutants were thus detectable. No extensive search for more effective supplements was made, but of those tried, none proved to be better. And it was noted that almost all mutants tested could survive and grow in this medium. The use of the " free pouring " technique permitted the use of very large numbers of replicates, with the elimination of chance errors and the consequent improved statistics. The multiple layer procedure also minimizes the contamination problem.

It should be pointed out that the layer technique produced a significant number of apparent " zero point " mutants which were not seen in the serial subculture technique. This is probably related to the slow diffusion of the streptomycin to allow a period of growth, even when no preincubation period is consciously permitted. In the course of pouring of very large numbers of plates, often as many as 1500 at a time, there is also some incubation in the hot laboratory before the streptomycin layer can be added. However, since the method depends on an end point reading which is stable for several hours, the zero point reading is of no interest, except to point out that there is no disagreement with DEMEREC's findings (1951).

Although mixed culture tests as suggested by LEDERBERG (1948) were not considered feasible because of the variety of mutant types, the inverse of that test, adding unirradiated non-mutant cells, provided the same kind of information. Little selection in either direction was observed.

SUMMARY

The development of a quantitative genetic method is described using the mutation of *E. coli* from streptomycin sensitivity to resistance. After an ex-

amination of the variables which affect the number of mutants detectable, a standardized technique evolved which provided reproducible information about the genetic effect of radiation.

Aliquots of irradiated cultures are imbedded in the middle agar layer of a triple layer plate consisting entirely of Difco Streptomycin Assay Agar (pH 8). After a 5-hour period of incubation at 37°C, a fourth agar layer which contains 500 μg per ml of streptomycin and a supplement of Difco Brain-Heart Infusion is added. The Petri dishes are then refrigerated overnight and finally incubated again for at least four days at 37°C before the surviving colonies are counted. Several different concentrations (below 5×10^8) of the culture must be treated in this manner and the value of the mutants per unit population plotted for each. The straight line which is obtained is extrapolated to the " y " axis and the intersect is taken as the mutation frequency for the culture. Non-irradiated controls, similarly treated, provide background values to be subtracted from each treatment.

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It is a pleasure to acknowledge the able technical assistance of MARY F. C. PERRY and FLORENCE Z. THANASSI.

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INCOMPATIBILITY IN AUTOTETRAPLOID TRIFOLIUM REPENS L. I. COMPETITION AND SELF-COMPATIBILITY [1]

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SELF-INCOMPATIBILITY is the inability of a plant producing functional male and female gametes to set seed upon self-pollination. The best known genetic system effecting incompatibility in plants is that in which a single series of oppositional alleles governs pollen-tube growth, with pollen behavior under autonomous, or gametophytic, genetic control (EAST and MANGELSDORF 1926). Self-incompatibility in diploid white clover, *Trifolium repens* L., was shown by ATWOOD (1940) to be conditioned by multiple oppositional (*S*) alleles of this type. The gametophytic *S* allele system is similarly operative in *T. pratense* (WILLIAMS and SILOW 1933), *T. hybridum* (WILLIAMS 1951; BREWBAKER 1951), and *T. nigrescens* (BREWBAKER, unpublished data).

Superimposed on the self-incompatibility system in most species are one or more mechanisms which reduce or inactivate pollen-tube inhibition, thereby permitting some self-fertilization to occur. One such mechanism occurring in polyploids is that of competition (LEWIS 1947). When certain oppositional alleles occur together in the heterogenic diploid pollen produced by autotetraploids, they interact (competition interaction) in such a way that the inhibition of these grains in self-pollinations is suppressed or inactivated. In *Trifolium repens* (ATWOOD and BREWBAKER 1953) and *T. hybridum* (BREWBAKER 1953) heterogenic grains that exhibited competition were never inhibited in self- or cross-pollinations, and functioned with a frequency equal to that of other uninhibited pollen classes in cross-pollinations. This type of self-fertility is intrinsically attributable to certain S allele combinations, and has been termed self-compatibility.

Self-compatible autotetraploids segregated in the F_1 family derived from two self-incompatible, colchicine-doubled, parental clones of white clover studied by ATWOOD (1944). The data from this and advanced generation families were interpreted on the hypothesis that competition occurred in one of the six heterogenic pollen classes (ATWOOD and BREWBAKER 1953). The present investigations were made in order to obtain more comprehensive information on the frequency and behavior of competition pollen classes and the resultant self-compatibility in autotetraploid white clover.

[1] Submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy, Cornell Univ.; Paper No. 304, Department of Plant Breeding, Cornell Univ., Ithaca, N. Y.

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MATERIALS AND METHODS

Parental diploid clones were derived largely from commercial seed lots of Oregon Certified Ladino clover and from white and Ladino clover stocks from the U. S. Regional Pasture Research Lab., State College, Pa. Colchicine-induced tetraploids were obtained from this material (BREWBAKER 1952b), and several additional diploid clones and their corresponding colchicine-induced tetraploids were kindly provided by DR. A. GERSHOY of the University of Vermont.

All plants were grown to maturity and pollinated in the greenhouse. Flowering heads were covered with muslin bags and hand-manipulated for self-pollination. Some of the self-pollinations were made by toothpick-tripping ten florets per head. The majority of the plants could be readily classified as self-incompatible or self-compatible on the basis of selfed seed sets from two heads.

All chi-square values were corrected for continuity by reducing the differences of observed and expected values by 0.5. Expected ratios were calculated on the basis of chromosome assortment, assuming complete linkage of the *S* locus and the centromere. Evidence has been obtained that the *S* locus in white clover does exhibit some crossing-over with the centromere (BREWBAKER 1952a). In the absence of a precise estimate of this map distance, chromosome assortment ratios were applied. Crossing over would alter the frequencies but not the kinds of segregating genotypes in the families studied.

EXPERIMENTAL RESULTS

Eleven diploid clones and the 11 corresponding 4N clones propagated from colchicine-doubled sectors were self- and cross-pollinated. All of the diploid clones were cross-compatible and self-incompatible (SI), setting an average of 0.7 seeds per head upon selfing. Eight of the 11 4N clones were similarly SI, averaging 0.8 selfed seeds per head. The three remaining 4N clones (B2, B11 and G1) were self-compatible (SC), and averaged 43.0, 30.1 and 59.7 seeds per head, respectively, following self-pollination.

Two of the three SC clones (B2 and G1) and seven of the eight SI clones were intercrossed in all combinations. Fifteen of the F_1 families and the two I_1 (selfed) families of B2 and G1 were grown, and from two to five heads on each of 600 fertile plants of these 17 families were self-pollinated. The types of progeny behavior in these families have been grouped as follows: (1) SC selfed → all SC, (2) SC × SC → 1 SI : 8 SC, (3) SI × SC → 1 SI : 2 SC, (4) SI × SI → 11 SI : 25 SC, and (5) SI × SI → all SI.

(1) *SC selfed → all SC*

Two inbred families were grown from selfed seed obtained on SC 4N clones G1 and B2. These two balanced diallelic plants were shown to differ in both *S* alleles by studies of F_1 and F_2 families of the corresponding diploids g1 and b2 (BREWBAKER 1952a). Clone G1 was symbolically designated $S_1S_1S_2S_2$ and clone B2, $S_3S_3S_4S_4$.

TABLE 1

SC selfed → all SC. Numbers and selfed seed averages of plants in two I_1 (selfed) families.

Family	Method of selfing	Number of plants	Seeds obtained upon selfing	
			Av.	Range in plant avs.
B2 I_1	Entire heads rubbed	29	15.9	4.0– 62.0
G1 I_1	Entire heads rubbed	17	30.1	8.7–106.5
	10 florets tripped	13	11.1	4.3– 19.0

All of the fertile plants in these two I_1 families were SC (table 1). One plant in each family was sterile. In addition, four inbred progeny of B2 produced indehiscent anthers. Inbred progeny of SC triallelic and tetraallelic plants of white clover reported by ATWOOD and BREWBAKER (1953) were similarly 100 percent SC, as were I_1 and I_2 families from SC 4N clones of alsike clover (BREWBAKER 1953). These results are explained by assuming that the heterogenic pollen grains of the competition type (e.g., S_1S_2 of clone G1) alone effect self-fertilization (table 2).

(2) *SC × SC → 1 SI : 8 SC*

SC clones G1 ($S_1S_1S_2S_2$) and B2 ($S_3S_3S_4S_4$) were crossed reciprocally following suction-emasculation, and 72 plants from the two crosses were tested for self-compatibility (table 3). The reciprocal F_1 families gave similar results and were grouped for final analysis. Ten SI and 62 SC plants were obtained, giving a satisfactory fit to a 1 SI:8 SC ratio ($\chi^2 = 0.176$, P = .70–.50).

The 1 SI:8 SC ratio was calculated on the assumption that only the balanced diallelic F_1 plants were SI, i.e., that none of the four heterogenic pollen classes arising *de novo* in the F_1 family (S_1S_3, S_1S_4, S_2S_3, and S_2S_4) were of the competition type (table 4). In order to obtain a critical test of this hypothesis, F_1 hybrids of the corresponding diploid cross, g1 (S_1S_2) × b2 (S_3S_4), were colchicine-treated. The five derived diallelic tetraploids obtained

TABLE 2

SC selfed → all SC. Expected classes in inbred family G1 I_1 ($S_1S_1S_2S_2$ selfed).

Female gametes	Classes expected from fertilization by following male gametes		
	1 S_1S_1	4 S_1S_2*	1 S_2S_2
1 S_2S_1	---	4 SC	---
4 S_1S_2*	---	16 SC	---
1 S_2S_2	---	4 SC	---

* S_1S_2 = competition pollen class.

TABLE 3

SC × SC → 1 SI : 8 SC. Numbers and selfed seed averages of plants in F_1 family G1 (SC) × B2 (SC).

Method of selfing	Number of plants	Seeds obtained upon selfing	
		Av.	Range in plant avs.
Entire heads rubbed	9 SI	2.1	0.0– 5.0
	44 SC	49.8	8.7–144.5
10 florets tripped	1 SI	1.5	....
	18 SC	7.3	2.5– 17.5
Total	10 SI : 62 SC	$\chi^2 = 0.176$, P = .70–.50*	

* Expected ratio = 1 SI : 8 SC (table 4).

from this treatment were SI. When four of these plants and three of the SI F_1 plants of G1 × B2 were intercrossed reciprocally, four intra-sterile, inter-fertile groups of 1, 1, 3 and 2 plants were obtained (BREWBAKER 1952a). The three SI 4N F_1 hybrids were represented in the first three of these groups. The four SI groups may be considered to represent the expected diallelic genotypes $S_1S_1S_3S_3$, $S_1S_1S_4S_4$, $S_2S_2S_3S_3$ and $S_2S_2S_4S_4$ (table 4). The self-incompatibility of these diallelic genotypes proved that, among the four homogenic and six heterogenic classes involving these four alleles, only the S_1S_2 and S_3S_4 pollen grains were capable of effecting self-fertilization.

(3) *SI × SC → 1 SI : 2 SC*

Six F_1 families were grown from crosses of SI clones with SC clones G1 and B2 (table 5). Five of these families segregated in nearly the 1 SI : 2 SC ratio expected if only F_1 plants which resulted from the functioning of competition gametes S_1S_2 or S_3S_4 of the SC male parents were SC (table 6).

One family (B4 × G1) segregated 20 SI and 14 SC plants, deviating significantly from the expected 1 SI : 2 SC ratio. This was the only family involving Ladino clover clone B4, which flowered and set seed poorly. The F_1 plants were not checked for male- or female-sterility which may have contributed to the disproportionately high number of SI segregants.

TABLE 4

SC × SC → 1 SI : 8 SC. Expected classes in F_1 family G1 × B2 ($S_1S_1S_2S_3 \times S_3S_3S_4S_4$).

Female gametes	Classes expected from fertilization by following male gametes		
	1 S_3S_3	4 S_3S_4*	1 S_4S_4
1 S_1S_1	1 SI	4 SC	1 SI
4 S_1S_2*	4 SC	16 SC	4 SC
1 S_2S_2	1 SI	4 SC	1 SI
Total	4 SI : 32 SC		

* S_1S_2 and S_3S_4 = competition pollen class.

TABLE 5

SI × SC → 1 SI : 2 SC. Numbers and selfed seed averages of plants in six F_1 SI × SC families.

Family	Number of plants	Seeds obtained upon selfing entire heads		χ^2 (P value)*
		Average	Range in plant avs.	
B1 × B2	20 SI	1.2	0.0– 4.7	0.049
	44 SC	39.2	6.5–108.0	(.90–.80)
G6 × B2	9 SI	0.3	0.0– 2.0	0.604
	26 SC	43.5	10.2–106.5	(.50–.30)
B1 × G1	17 SI	0.1	0.0– 3.0	0.021
	37 SC	42.1	9.0– 84.0	(.90–.80)
B4 × G1	20 SI	0.4	0.0– 2.0	8.826
	14 SC	24.4	7.0– 68.0	(< .01)
G4 × G1	6 SI	0.7	0.0– 1.3	3.092
	28 SC	44.7	5.2–110.5	(.10–.05)
G6 × G1	11 SI	1.0	0.0– 4.0	0.004
	24 SC	55.8	9.5–171.0	(.95)

* Expected ratio = 1 SI : 2 SC (table 6).

The cross of G4 × G1 segregated 6 SI : 28 SC plants (table 5). This observed segregation was almost identical with the 7 SI : 29 SC ratio expected on the assumption that, in addition to the competition gamete of the male parent, one of the heterogenic gametes appearing *de novo* in this F_1 family was of the competition type. The deviation of observed data from the 1 SI : 2 SC ratio approached significance (P = .10–.05), and suggested the appearance in this family of a second heterogenic competition class involving allele S_1 or S_2 of clone G1.

The SI progeny from crosses of SI × SC balanced diallelics are expected to comprise four diallelics and two triallelics in a ratio of 1 : 1 : 1 : 1 : 4 : 4 (table 6). Fifteen of the SI plants in family B1 × G1 were genotypically classified by backcrosses and F_1 intercrosses (BREWBAKER 1952a). The observed 2 : 1 : 1 : 0 : 8 : 3 segregation fitted the expected ratio satisfactorily.

TABLE 6

SI × SC → 1 SI : 2 SC. Expected classes in F_1 family B1 × B2 ($S_3S_5S_6S_6 \times S_3S_3S_4S_4$).

Female gametes	Classes expected from fertilization by following male gametes		
	1 S_3S_3	4 S_3S_4*	1 S_4S_4
1 S_5S_5	1 SI	4 SC	1 SI
4 S_5S_6	4 SI	16 SC	4 SI
1 S_6S_6	1 SI	4 SC	1 SI
Total	12 SI : 24 SC		

* S_3S_4 = competition pollen class.

TABLE 7

SI × SI → 11 SI : 25 SC. Numbers and selfed seed averages of plants in two F_1 SI × SI families and one derived diallelic family.

Family	Number of plants	Seeds obtained upon selfing entire heads	
		Average	Range in plant avs.
G6 × G7	15 SI	1.4	0.0– 4.0
	43 SC	51.0	10.0–203.5
G6 × B6	2 SI	2.0	1.0– 3.0
	9 SC	85.1	29.0–196.0
(g6 × b6) + c-trt.	3 SI	0.2	0.0– 0.5
	4 SC	130.4	49.5–199.0

(4) *SI × SI → 11 SI : 25 SC*

Two F_1 families (G6 × G7, G6 × B6) from SI 4N parents segregated SC and SI plants (table 7). Family G6 × G7 segregated 15 SI and 43 SC plants, which fitted a ratio of 11 SI : 25 SC ($\chi^2 = 0.401$, P = .70–.50). The 11 SI : 25 SC ratio is calculated upon the occurrence of a single competition class among the four heterogenic classes which arise *de novo* in the F_1 of SI balanced diallelics (table 8). This type of segregation was observed in the original study of incompatibility in white clover autotetraploids (ATWOOD 1944).

Family G6 × B6 comprised 2 SI and 9 SC plants. In addition, the corresponding diploid cross g6 × b6 was made, and some of the F_1 seedlings were colchicine treated. All of the 2N F_1 plants were SI. Seven tetraploids (derived diallelics) were obtained from colchicine-treated g6 × b6 seedlings. Three of the seven were SI, and the four remaining were SC (table 7). If one of the four heterogenic pollen classes unique to the F_1 progeny of G6 × B6 were of the competition type, the F_1 would be expected to segregate 11 SI : 25 SC. Similarly, derived diallelic 4N plants obtained from colchicine-treated g6 × b6 seedlings would be expected to segregate 3 SI : 1 SC. However, if two of the four heterogenic pollen classes were of the competition type, a ratio of 1 SI : 5 SC or of 1 SI : 17 SC would be expected for the 4N F_1 progeny of

TABLE 8

SI × SI → 11 SI : 25 SC. Expected classes in F_1 family G6 × G7 ($S_9S_9S_{10}S_{10} \times S_{11}S_{11}S_{12}S_{12}\lambda$

Female gametes	Classes expected from fertilization by following male gametes		
	1 $S_{11}S_{11}$	4 $S_{11}S_{12}$	1 $S_{12}S_{12}$
1 S_9S_9	1 SI	4 SC*	1 SC*
4 S_9S_{10}	4 SI	16 SC*	4 SC*
1 $S_{10}S_{10}$	1 SI	4 SI	1 SI
Total	11 SI : 25 SC		

* Recombination gamete genotype S_9S_{12} assumed to be competition pollen class.

G6 × B6, with a ratio of 1 SI : 1 SC for the 4N derived diallelics. The observed data (2 SI : 9 SC for G6 × B6, 3 SI : 4 SC for diallelics derived from g6 × b6) were too limited to distinguish statistically between the alternative hypotheses, although the latter was favored.

(5) *SI × SI → all SI*

Six F_1 families which were obtained from crosses of SI colchicine-doubled parents comprised only SI plants. The 144 F_1 plants averaged 0.8 selfed seeds per head (table 9). The absence of SC segregants indicated that no competition pollen classes had appeared among the 24 heterogenic gametes (maximum estimate) arising *de novo* in these 6 F_1 families. Genotypic classification of 18 and 39 plants, respectively, in SI × SI families G4 × G6 and G4 × B1 confirmed the expected segregations of four diallelic, four triallelic, and 1 tetraallelic genotypes (BREWBAKER 1952a).

TABLE 9

SI × SI → all SI. Numbers and selfed seed averages of plants in six SI × SI families.

Family	Number of plants	Seeds obtained upon selfing entire heads	
		Average	Range in plant avs.
B1 × G4	39	1.1	0.0–6.0
B1 × G6	38	1.0	0.0–5.0
B1 × G7	20	0.4	0.0–3.0
G4 × G6	22	0.7	0.0–5.0
G4 × G7	7	0.1	0.0–1.0
G6 × B7	18	0.5	0.0–3.0

DISCUSSION

The experimental data obtained in this study permit the extension of the hypotheses of ATWOOD and BREWBAKER (1953) to a large number of *S* allele genotypes in *Trifolium repens*. The results confirm the hypotheses that (a) homogenic pollen classes produced by autotetraploids are inhibited in self-pollinations, (b) only certain heterogenic pollen classes are capable of effecting self-fertilization, and (c) these heterogenic (competition) pollen grains function in all cross-pollinations, and with a frequency equal to that of other uninhibited grains.

The competition of S alleles in heterogenic pollen grains appears to be such that the pollen substances produced by certain alleles when they occur separately (in haploid or homogenic diploid pollen) are not fully elaborated or interact in such a way as to render them ineffective when the alleles occur together in heterogenic pollen grains. An interesting parallelism may be drawn between the genetic indifference of competition pollen (i.e., its ability to effect fertilization on plants of all *S* genotypes) and the genetic indifference of certain tetraploid ascites tumors in mice (HAUSCHKA and LEVAN 1953). Whereas diploid ascites tumors are completely strain-specific, tumors with

tetraploid modality exhibit varying degrees of genetic indifference to host lines. The tetraploid tumors appear to lack sufficient antigen to evoke the protective antibody titer in susceptible mice strains (HAUSCHKA and LEVAN 1953). The report by LEWIS (1952) that S alleles produce antigenically distinct pollen substances suggests that competition pollen classes similarly lack sufficient " antigen " to be inhibited on styles of all *S* genotypes.

The frequency of occurrence of the competition type of S allele interaction in heterogenic pollen grains of 4N white clover may be estimated from the data presented. A total of 71 different heterogenic *S* allele combinations (maximum estimate) were studied. Eleven of these occurred in the colchicine-doubled parents, and 60 arose as the result of recombination in the 15 F_1 families. These values are calculated on the assumption that the 11 parental clones had no alleles in common. The results of ATWOOD (1942) suggest that this may be validly postulated. All genotypic evidence obtained from 2N and 4N hybrid families of these clones supported this postulate. Three of the 11 parental 4N clones were SC, indicating that three of the 11 parental heterogenic gametes were of the competition type. Among the 60 heterogenic gametes of the 15 F_1 families, one in family G6 × B6 and one in family G6 × B7 were also of the competition type. It is suggested that two additional heterogenic gametes—one occurring in family G4 × G1, and one in family G6 × B6—may be included in this group. In summary, a maximum of five (or seven) of the 71 heterogenic *S* allele combinations functioned to effect self-fertilization by means of competition.

This observed frequency of 8–10 percent competition types among total heterogenic gametes tested in white clover is in general agreement with that obtained in alsike clover (BREWBAKER 1953), in which two (5 percent) of the 39 heterogenic classes tested were of the competition type. A similar frequency appears to obtain in 4N red clover.

Competition gamete frequencies of the order of magnitude observed would result in advanced generation populations derived from colchicine-doubled plants in which a large proportion of the plants would be self-compatible. It may be calculated, for example, that a random F_1 hybrid population of the 11 colchicine-doubled white clover plants studied here would comprise ca. 36 percent SC plants. This calculation is made on the assumption that, of the 231 possible heterogenic combinations of the 22 different alleles, only five (those clearly indicated among the 71 tested) were of the competition type. If it is assumed that 20 of the 231 (thus approximating the observed frequency of 9 percent) were competition gametes, three of which appeared in the 11 parental clones, the F_1 population would comprise ca. 47 percent SC plants. These empirical calculations are supported by selfing data from Scandinavian strains of 4N alsike clover, in which over 50 percent of the plants are fully self-compatible (BREWBAKER, unpublished data).

In crop plants such as the true clovers, the degree of inbreeding with consequent depression of vigor incurred in such populations might prove a serious deterrent to the establishment of tetraploid varieties that are sufficiently vig-

orous and high-yielding to warrant recommendation. However, the competition mechanism conditioning self-compatibility is of such nature that self-compatibility could be eliminated at any stage in a 4N breeding program (ATWOOD and BREWBAKER 1953).

SUMMARY

1. Eleven self-incompatible (SI) diploid clones of *Trifolium repens* were studied. Diallel intercrosses of these clones confirmed the operation of oppositional *S* alleles conditioning self-incompatibility in this species.

2. Of the eleven corresponding colchicine-doubled clones, three were self-compatible (SC), setting an average of 44.3 selfed seeds per head. The eight remaining 4N clones were SI, averaging 0.8 selfed seeds per head.

3. The frequencies of SI and SC 4N plants were determined in 15 F_1 and two I_1 families comprising 600 plants. The observed family patterns were of five primary types: (a) SC selfed → all SC, (b) SC × SC → 1 SI : 8 SC, (c) SI × SC → 1 SI : 2 SC, (d) SI × SI → 11 SI : 25 SC, and (e) SI × SI → all SI.

4. The observed frequencies of SC plants in these families were in accord with the hypotheses that (a) self-compatibility results from the uninhibited growth in self-pollinations of pollen tubes possessing specific S allele combination, and that (b) such heterogenic pollen grains (competition class) are never inhibited and function in cross-pollinations with a frequency equal to that of other uninhibited gametes.

5. A total of five (or seven) of the 71 (maximum estimate) different heterogenic *S* allele combinations tested were of the competition class. This frequency (8–10 percent) of competition gametes among all possible heterogenic *S* allele combinations is calculated empirically to effect nearly 50 percent self-compatibility among plants in advanced generations of the eleven colchicine-doubled clones studied.

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DOMINANT LETHAL AND INACTIVATION EFFECTS OF NITROGEN MUSTARD ON HABROBRACON SPERM

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AS early as 1911 O. HERTWIG demonstrated two types of behavior of irradiated Amphibian sperm. One of these, the dominant lethal effect, resulted in death of embryos due to fusion of injured sperm nucleus with egg nucleus. The other, inactivation of sperm, stimulated the egg to develop gynogenetically since injured sperm entered it but took no part in development. PACKARD (1914) observed, in addition to these phenomena, a second type of inactivation in which heavily irradiated Nereis sperm failed to penetrate the egg. With this type likewise, eggs were activated to develop gynogenetically. MULLER (1927) used the partial sterility of irradiated Drosophila males as evidence "for the first time, of the occurrence of dominant lethal genetic changes, both in the X and in the other chromosomes."

The parasitic wasp *Habrobracon juglandis* was used for the present study. In the cross utilized about 62% of the eggs are fertilized and develop into diploid females; 38% are not fertilized and develop parthenogenetically into haploid males. In this type of reproduction dominant lethal effects can be distinguished from sperm inactivation without cytological study. Any treatment of sperm which reduces hatchability at the expense of females, leaving number of males unchanged, has induced dominant lethal effects only. When hatchability is reduced to 38% and all survivors are males, there has been induced in each sperm at least one dominant lethal. When, after the attainment of complete dominant lethality, hatchability rises above 38% and all survivors are males, some sperm have been inactivated and are either entering the eggs without taking part in development or are failing to enter them. These facts are contrasted with expectation in a form like Drosophila in table 1. In making tests for distinguishing dominant lethal effects from inactivation, an advantage lies in the fact that polyspermy is rare in Habrobracon. About 1% of fertilized eggs have two sperm in them. Almost every fertilized egg therefore represents a test of a single sperm so that masking of inactivation by dominant lethality occurs rarely.

STANCATI (1932) recognized the possibility of inactivation and the fact that it could be identified readily in Habrobracon but showed conclusively that after exposure of males to 2500 r, dominant lethal changes only were

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induced. MAXWELL (1938), P. W. WHITING (1938) and HEIDENTHAL (1945) demonstrated that at X-ray doses above 10,000 r some sperm are inactivated as demonstrated by the fact that survival ratio increases after having fallen to 33% at lower doses. Recently (1953) BROWN and CAVE have shown that, due to dominant lethals, after the application of 2000–4000 r to pollen of *Lilium formosarum*, few or no seeds are set. In competition with untreated pollen, treated grains are not handicapped in ability to achieve fertilization. All investigators agree in finding no inactivation until after the attainment of complete dominant lethality.

TABLE 1

Theoretical expectations for hatchability percentages and sex ratios of adult survivors for Drosophila and Habrobracon under different conditions of dominant lethality and sperm inactivation.

Conditions	% hatchability	Adult survivors	
		% ♂♂	% ♀♀
Controls			
Drosophila	100	50	50
Habrobracon	100	38	62
50% dominant lethals			
Drosophila	50	50	50
Habrobracon	69	55	45
100% dominant lethals			
Drosophila	0	0	0
Habrobracon	38	100	0
100% dominant lethals + 50% inactivation			
Drosophila	0	0	0
Habrobracon	69	100	0
100% dominant lethals + 100% inactivation			
Drosophila	0	0	0
Habrobracon	100	100	0

Drosophila: lethals are autosomal. Habrobracon: 62% of eggs are fertilized.

The present study is concerned with the effects of nitrogen mustard, methyl bis (beta chloroethyl) amine hydrochloride [2], on Habrobracon sperm and the sorting of dominant lethal effects from those due to inactivation. Whether the sperm inactivation observed is the HERTWIG type (lack of pronuclear fusion) or that described by PACKARD (lack of egg penetration) or a third kind in which sperm are completely immobile has not been determined. This is being investigated cytologically at present.

MATERIALS AND METHODS

Mature unmated males or males separated from females for several days were used. They were exposed to an aerosol of 10% aqueous solution in

[2] Provided by Merck & Company.

alternating exposures of 50 seconds aerosol and 10 seconds air with time as the variable. During the 50-second interval 4.7 liters of aerosol were delivered to the exposure chamber containing the wasps and 4.2 liters of air were delivered during the 10-second interval. The aerosol was produced by a De-Vilbiss-40 glass nebulizer. The flow of air served to dilute the atmosphere and to evaporate coalesced droplets of the mutagenic solution which may have adhered to the animals or walls of the exposure chamber. The males were then kept for at least twenty-two hours before mating. This delay in mating was found to be necessary because of the mutagenic effect on eggs of the nitrogen mustard carried by freshly exposed males. In most instances matings

TABLE 2

Single matings of males after exposure to nitrogen mustard.

Exposure in minutes	% Hatchability				Progeny		% inactivation
	1st day	1st 5 days	6–Days	Total	♂♂	♀♀	
2.5	$\frac{34}{138}=24.64$	$\frac{120}{524}=22.90$	$\frac{105}{404}=25.99$	$\frac{225}{928}=24.25\pm1.41$	163	0	0
5	$\frac{43}{171}=25.15$	$\frac{317}{957}=33.12$	$\frac{570}{1519}=37.52$	$\frac{887}{2476}=35.82\pm0.96$	612	1	0
10	$\frac{29}{57}=50.88$	$\frac{170}{437}=38.90$	$\frac{237}{622}=38.10$	$\frac{407}{1059}=38.43\pm1.49$	377	0	0
20	$\frac{47}{99}=47.47$	$\frac{239}{561}=42.60$	$\frac{281}{665}=42.26$	$\frac{520}{1226}=42.41\pm1.41$	441	0	7.11
30	$\frac{34}{70}=48.57$	$\frac{51}{115}=44.35$		$\frac{51}{115}=44.35\pm4.63$	42	0	10.24
60	$\frac{140}{243}=57.61$	$\frac{329}{558}=58.96$		$\frac{329}{558}=58.96\pm2.08$	119	1	33.81
90	$\frac{49}{69}=71.01$	$\frac{284}{399}=71.18$		$\frac{284}{399}=71.18\pm2.27$	113	0	53.52
Controls							
0	$\frac{22}{22}=100$	$\frac{191}{196}=97.45$	$\frac{139}{142}=97.89$	$\frac{330}{338}=97.63\pm0.83$	101	265	

were observed and males were removed after a single mating. Females were placed individually in small Stender dishes, each of which contained one host Ephestia larva. After six to eight hours the females were removed and kept overnight without the host and were given a fresh host larva the following morning. This was repeated as long as desired. Eggs are deposited on the surface of the paralyzed host and are easily counted. Records were taken of number laid at time of removal of females and of number of larvae after a forty-eight-hour period at 30°C. The six- to eight-hour period of oviposition has been found best as it allows time for the laying of a number of eggs convenient for observation and counting with no wide duration in time of hatching. Larvae were permitted to mature in order that sex of survivors could be recorded.

Stocks with different sex alleles were used as sources of males and of females in order to avoid the production of highly inviable diploid males characteristic of crosses involving two sex alleles. The males were from virgin mothers or were carefully checked in order to insure that they were not sterile diploids. Hatchability of eggs from the type of cross used in this study is about 98%.

RESULTS

Data from single matings of exposed males

The data from single matings of exposed males are summarized in table 2. When males appeared to mate normally but hatchability of eggs was about 100%, it was taken for granted that males had no sperm or that mating had not been completed and data are not included in summaries. It will be seen

TABLE 3

Repeated matings of males exposed for 2.5, 20, and 30 minutes and, for postponed mating of males exposed for 20 minutes.

Exposure in minutes	Date of exposure	Date of mating	% hatchability	Progeny	
				♂♂	♀♀
2.5	11/26	11/27	$\frac{225}{928} = 24.25 \pm 1.40$	163	0
2.5	11/26	11/28	$\frac{89}{344} = 25.87 \pm 2.36$	68	0
20	9/22	9/23	$\frac{356}{821} = 43.36 \pm 1.73$	305	0
20	9/22	9/25	$\frac{195}{395} = 49.37 \pm 2.52$	182	0
20	9/22	9/26	$\frac{185}{342} = 54.09 \pm 2.69$	146	0
20	9/22	10/3	$\frac{42}{102} = 41.18 \pm 4.87$	27	0
20	9/22	10/6	$\frac{28}{64} = 43.75 \pm 6.20$	16	0
30	5/13	5/14	$\frac{51}{115} = 44.35 \pm 4.63$	30	0
30	5/13	5/25		121	6
20	9/22	9/25	$\frac{54}{107} = 50.47 \pm 4.83$	38	0

that even the lightest treatment, that of two and one-half minutes, induced at least one dominant lethal in each sperm since no females were produced. Results of this particular experiment are anomalous in one respect and in this are not comparable with any of those obtained during many years of study of Habrobracon. Hatchability is significantly lower than expectation, which is about 38%. One might suggest that matings were made too soon after ex-

posure of males to nitrogen mustard so that injury induced in eggs increased mortality, but these males were handled as were those receiving longer exposure; reference to table 3 discloses that subsequent matings of these males gave almost identical lowered hatchability. This will be discussed later.

Five- and ten-minute exposures appear to induce dominant lethal effects only. Hatchability percentages, 35.8 and 38.4, are not significantly different from expectation, 38%. With the twenty-minute exposure a barely significant increase in hatchability is apparent. This increase becomes more and more significant with each extension of length of treatment until at ninety minutes a hatchability of 71.7% is attained. Theoretically, after a dose of about two hundred minutes, hatchability should be 98%, but males would probably not

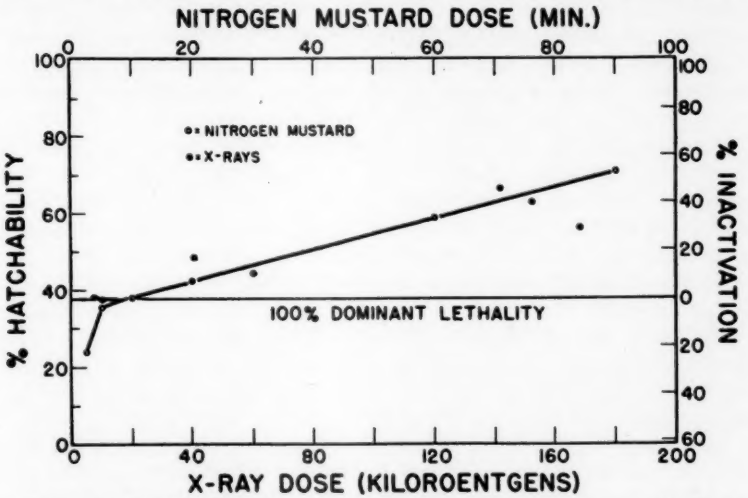

FIGURE 1.—Egg hatchability showing sperm inactivation after exposure of males to nitrogen mustard or to X-rays; data superimposed. X-ray data from MAXWELL (1938) and HEIDENTHAL (1945). Doses are lethal or higher than lethal.

tolerate an exposure of this length. In the ninety-minute experiment the aerosol and air were delivered at a slightly lower rate of flow than in the other experiments. A lower rate would consequently decrease the dose, so the true inactivation at ninety minutes may be somewhat higher than shown in table 2. Since ninety minutes was very near the lethal dose for the wasps, there was no repetition at the higher rate of flow used in all other experiments.

Percentages of inactivation can be estimated by subtracting from actual hatchability that expected if dominant lethals only were induced, that is, 38%, and dividing the result by the expected percentage of fertilized eggs, in this case 62. These percentages of inactivation as well as those of hatchability are plotted in figure 1.

When females are mated they receive only mature sperm. Control females,

after a single mating, may continue to produce daughters for from three to four weeks. Normal sex ratio is maintained until sperm are exhausted when there is a sudden cessation of female production. Since mature males, either unmated or kept from females for several days, were exposed to nitrogen mustard, an ample supply of mature sperm must have been present at time of exposure. The holding of such males for about twenty-two hours before mating would not be expected to result in the addition of many sperm matured from earlier stages at time of exposure even should such stages prove resistant to the treatment.

Tests of hatchability over a period of time after the single mating of treated males answer several questions if they are carried on for a long enough time. Should hatchability rise suddenly to 98% with all progeny male, all sperm will have become exhausted. Should hatchability fall to 38% with all progeny male, sperm will have recovered from inactivation. Should hatchability rise and females appear or increase among progeny, sperm will have recovered from injury inducing dominant lethal effects.

Reference to table 2 shows that there has been no significant change in hatchability within the period covered by the tests which, in some cases, were continued for twenty-one days after the single mating. In no female among the one hundred and three used was the sperm supply exhausted. The appearance of two females among the progeny, one in the five-minutes and one in the sixty-minute experiments, can be explained perhaps by chance escape of an occasional sperm from injury or the rare maturation of a functional sperm from a more resistant earlier stage in meiosis during the twenty-two-hour period between exposure and mating. The fact that the females appeared late in the experiments, on the 8th and 19th day after the matings, may favor the idea of some slight recovery.

Frequently females of these experiments were set in vials after hatchability-record-taking was discontinued and allowed to reproduce as long as they survived. The two F_1 females mentioned above were the only ones produced in a total of 3798 progeny. Females mated to males exposed to the lowest doses, two and one-half and five minutes, produced 764 males, 0 females, and 728 males and 1 female respectively.

Data from repeated matings of exposed males

Another question which arises concerns the nature of the effects of nitrogen mustard on cells in earlier stages of meiosis at time of exposure. In table 3 are recorded some data from successive matings of exposed males and from one experiment in which two males were mated for the first time three days after exposure. Data are not as extensive as would be desirable. In the series exposed for twenty minutes and then mated one, three, four, eleven and fourteen days after exposure, hatchability remains constant with the exception of the four-day test where the fact that one female exhausted her supply of sperm increased hatchability.

In the two tests of the two and one-half minute exposure there is, again,

no evidence of any change in sperm behavior. The low hatchability of both groups for this dose is to be noted. Data for the thirty-minute experiments are given, although hatchability records were taken in one only, because of the appearance of six females from the mating made twelve days after the exposure. The consistently late appearance of all females is to be noted. The supply of mature sperm in males which have not mated must be large since control males may mate as many as fourteen times in rapid succession and produce daughters in every mating. All tests herein described may, therefore, deal with sperm mature at time of exposure.

All data obtained agree in giving no convincing evidence for recovery of exposed sperm from either dominant lethal or inactivation effects. Neither is there evidence for augmentation of either effect in the exposed sperm whether stored in the male or the female. The data suggest, in addition, that sperm remain "functional" as long as do those in control females after a single mating.

DISCUSSION

The dose of X-rays inducing at least one dominant lethal in every sperm is approximately the same for Habrobracon (P. W. WHITING 1938; HEIDENTHAL 1945) and for the frog (RUGH, 1939). HEIDENTHAL obtained one female among 1106 progeny after exposure of males to 10,000 r and RUGH found that only 1.6% of eggs hatched after exposure of frog sperm to this dose. Both reported that as dose was increased above this, hatchability increased. Inactivation of sperm, then, followed after the attainment of 100% dominant lethality.

In figure 1, X-ray data on inactivation are plotted for comparison with nitrogen mustard effects. In order to obtain a dominant lethal curve for Habrobracon sperm after exposure to nitrogen mustard for comparison with that induced by X-rays, much shorter exposures, or probably better, much more dilute solutions, should be used.

The anomalous hatchability ratios of eggs from females mated to males exposed to nitrogen mustard for two and one-half minutes are difficult to explain. Actually, from first matings of such males, progenies from eleven females were tested. Of these, four gave hatchabilities not significantly different from expectation, 32.4, 33.3, 34.0 and 38.0%. The remaining seven gave percentages ranging from 8.3 to 25. Subsequent tests of four of these males selected at random gave 19.3, 21.6, 28.7 and 29.3% respectively. These differ significantly from expectation of 38%. No convincing explanation of this comes to mind, but it appears that the treated sperm have been "stimulated" to fertilize more eggs than do the untreated sperm. The effect has never been observed after X-ray treatment.

The nature of sperm inactivation in Habrobracon is still unknown. Theoretically, sperm could enter the egg but take no part in development, or they could be active but not enter the egg or, in a form with natural parthenogenesis such as this, they could be inactive literally, moving little or not at all.

MACBRIDE (1946) found that although sperm of sterile diploid Habrobracon males are motile, penetration of the eggs does not occur. A similar study is now being conducted on sperm of heavily irradiated males.

AUERBACH and ROBSON (1947) mentioned that no histological study had been made of the effects of nitrogen mustard on the testes of Drosophila. Mature sperm remained motile and induced dominant lethal effects after exposure. Extensive breeding tests indicated an inhibition of spermatogenesis and absence of mature sperm after exhaustion of supply present at time of exposure. Tests herein reported are not extensive enough to permit the formation of conclusions in respect to inhibition of meiosis.

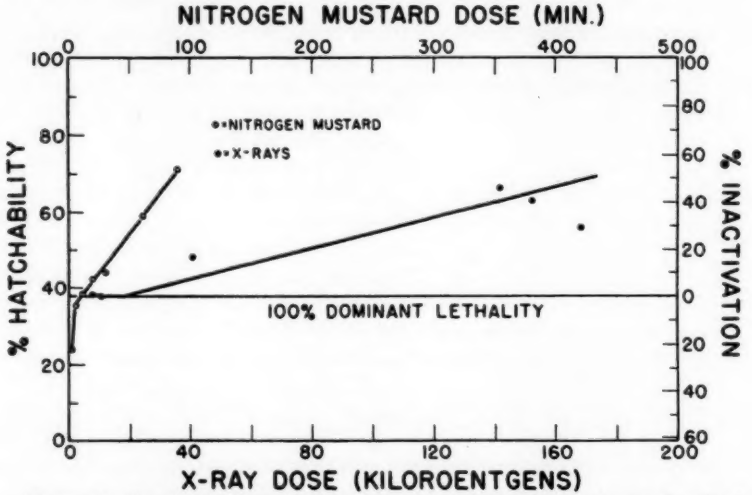

FIGURE 2.—Egg hatchability showing sperm inactivation after exposure of males to nitrogen mustard or to X-rays; relationship of sensitivities arranged on basis of comparison with egg sensitivities. Sperm five times as resistant to X-rays as to nitrogen mustard. X-ray data from MAXWELL (1938) and HEIDENTHAL (1945). Doses are lethal or higher than lethal.

ANNA R. WHITING (1945) has demonstrated that approximate lethal X-ray doses are 2000 r for eggs in first meiotic metaphase and 50,000 r for those in first meiotic prophase. The approximate lethal dose for sperm, as pointed out above, is 10,000 r. Lethal dose of nitrogen mustard for eggs in first meiotic metaphase is about 8–10 minutes. Eggs treated in the first meiotic metaphase and first meiotic prophase show the same relative sensitivity whether treated with nitrogen mustard or X-rays (WHITING and VON BORSTEL 1952). On this basis the lethal dose for sperm after nitrogen mustard treatment should be 40–50 minutes. Actually, the lethal dose for sperm is two and one-half minutes or less. Figure 2 compares sperm inactivation induced by nitrogen mustard with that induced by X-rays when egg metaphase and

prophase sensitivity are used as criteria. The idea presents itself that small size of sperm cells and their position in the body permit greater accessibility of chromosomes to the mutagen than is true of the relatively large yolk-laden eggs. Relative sensitivities derived from X-ray studies would be expected to be more accurate than those made with nitrogen mustard.

SUMMARY

Data are presented which show that nitrogen mustard, methyl bis (beta chloroethyl) amine hydrochloride, produces a dominant lethal effect in every sperm in the shortest time during which mature males of the parasitic wasp Habrobracon were exposed. As length of exposures was increased beyond lethal dose, increased sperm inactivation occurred. This is expressed as an increase in parthenogenetic development and haploid male production due either to failure of sperm to penetrate eggs or to undergo syngamy if they enter. There is no evidence for recovery of sperm from either of these effects.

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THE GENERAL INCIDENCE OF PSEUDO-WILD TYPES IN NEUROSPORA CRASSA

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IN haploid organisms such as Neurospora wild-type progeny from crosses involving linked mutants in repulsion are usually interpreted as resulting from recombination. However, in a study of certain pyrimidine-requiring mutants, it has recently been shown that phenotypically wild-type strains found in the progeny of mutant × mutant crosses were not the result of crossing over nor could they readily be explained as resulting from back mutation or suppression of the mutant character (MITCHELL, PITTENGER and MITCHELL 1952). Crosses between these phenotypically wild-type strains and standard wild-type strains gave approximately 50% mutant progeny. Such strains have been called pseudo-wild types (PWT). A large number of different PWT's recovered from the progeny of mutant × mutant crosses were wild types insofar as their ability to grow on minimal medium was concerned. All gave from 40 to 50% mutant progeny when outcrossed to standard wild-type strains, and both mutants of the parental strains were recovered in the outcross progeny providing a translocation involving the pyrimidine (D) linkage group was not present. However, if a translocation linked to the *pyr* mutants was present in one of the strains involved in a cross from which PWT's were isolated, then either one or the other, but never both, of the parental mutants was recovered in the outcross progeny of any one PWT. PWT strains involving the *pyr* mutants were usually found by plating random spores from mutant × mutant crosses and selecting wild-type progeny. However, PWT's were also recovered from individual asci and such asci contained either four adjacent normal spores and four adjacent aborted spores or six normal and two aborted spores.

The behavior of these PWT's is consistent with the view that they are heterozygous disomic strains resulting from non-disjunction of the chromosome pair carrying the *pyr* mutants if it is assumed that nuclei with n + 1 chromosomes are unstable in mitotic divisions and give rise to heterocaryons containing both haploid and disomic nuclei and that only the haploid nuclei function in fertilization. In some respects PWT's behave as if they arose from heterocaryotic ascospores, but the different arrangements of the normal and aborted spores within the asci from which PWT's were recovered, the normal size of the spores from which they were derived, and the fact that both original

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mutants of the parental strains were not recovered when a translocation was involved are not consistent with the view that PWT's arose from heterocaryotic ascospores.

The purpose of the studies to be reported here was (1) to determine whether the phenomenon giving rise to PWT strains in Neurospora was due to some inherent properties of the D linkage group or whether PWT's could be found involving other linkage groups, (2) to describe the characteristics of any new PWT's, and (3) to obtain additional evidence on the origin of PWT's.

METHODS AND MATERIALS

The method used in selecting PWT strains in *Neurospora crassa* was based on the assumption that a disomic strain heterozygous for non-allelic recessives genes in repulsion would have a wild phenotype and would segregate for mutant progeny when outcrossed to standard wild-type strains. If phenotypically wild-type strains were selected from crosses between closely linked mutant genes, one should be able to distinguish PWT's from recombinant wild types by outcrossing them to standard wild-type strains since the PWT's should segregate for mutant progeny whereas recombinant types would not segregate.

Closely linked or possibly allelic genes were selected from each of five different linkage groups in which mutants for such a study were available. Crosses were made between selected linked mutants, and ascospores from these crosses were collected from the walls of the test tubes in which the crosses had been made. Spores were plated on minimal agar medium in Petri dishes, usually at concentrations of 1000–2000 spores per plate, heat activated at 60°C for 30–40 minutes, and incubated overnight at either 25°C or 35°C depending upon whether or not a temperature sensitive mutant was present. Ten to fifteen hours after heat activation the plates were examined under low magnification and the frequency of wild-type progeny was determined. In most cases germinating wild-type and mutant ascospores can readily be distinguished by differences in their growth on minimal medium since wild-type spores germinate and continue to grow whereas mutant spores germinate but the hyphae either do not grow or grow very little (LEIN, MITCHELL and HOULAHAN 1948; HAAS, MITCHELL, AMES and MITCHELL 1952). Individual wild-type and subwild-type spores (those intermediate between wild and mutant in their growth on minimal medium) which were completely isolated on the plates were removed and placed in small vials of minimal medium. Those strains whose wild phenotype was confirmed by their ability to grow in liquid minimal medium were outcrossed to standard wild-type strains. Mature ascospores from these crosses were then plated on minimal medium as described above. If all of the progeny of a given cross were wild, the particular wild-type was scored as a recombinant wild type. If, however, approximately 50% of the progeny were mutants, the strain being tested was classified as a PWT and saved for further study.

When albino mutants were used in crosses to look for PWT's, a somewhat different method of detecting wild-type progeny was employed since the wild and albino strains cannot be distinguished by differences in their growth on minimal medium. In this method double mutants of albino strains with a colonial temperature mutant, *co-t* (MITCHELL and MITCHELL 1952) were used. *Co-t* strains developing from ascospores form small button-like colonies approximately 1–2 mm in diameter after five days growth at temperatures over 31°C, but are wild type in their growth at temperatures below 31°C. Ascospores from crosses between two albino strains, both carrying the *co-t* mutant, were incubated at 35°C for five days. At that time the frequency of wild (pink) colonies and mutant (white) colonies was determined and all of the wild types which were sufficiently isolated on the plates were removed and later tested to determine whether they were PWT's or recombinant wild types.

The breeding behavior of possible PWT's was studied by determining the frequency of wild and mutant progeny in crosses with standard wild-type strains and occasionally in crosses with one of the mutant parents. Several hundred germinating ascospores from the outcross progeny of each PWT and a standard wild-type strain were classified as wild or mutant according to their growth on minimal agar plates. In all crosses of PWT × wild some of the segregants classified as mutants on the plates were transferred to vials of complete medium and later tested to verify the classification of mutants on the plates, and also to determine whether both of the parental mutants were present among the mutant segregants.

In order to determine whether or not mutant strains could be isolated from PWT strains, a dilute suspension of conidia from such strains was plated on minimal agar plates and individual conidia which had germinated but showed little other evidence of growth were transferred to complete medium and later tested to determine whether any mutant strains were present among those isolated.

Additional tests were carried out to determine whether or not PWT's were stimulated in their growth when the medium was supplemented with the growth requirements of the mutants present in the PWT's. PWT strains were grown at either 25°C or 35°C in 20-ml liquid cultures of minimal medium and minimal medium supplemented with the growth requirements of the mutants involved. After four days growth the mycelium was removed and dry weight measurements taken.

EXPERIMENTAL RESULTS

Crosses were made between selected closely linked or possibly allelic mutants from five different linkage groups and the frequency of phenotypically wild-type progeny was determined by classifying viable ascospores from these crosses on minimal agar plates. The number of phenotypically mutant and wild-type progeny from 20 different crosses as well as the number of PWT's which were isolated from these crosses are given in table 1. Ninety-nine PWT

TABLE 1

A list of the crosses which were made in this investigation together with the number of viable progeny classified and the number of phenotypically wild-type and pseudo-wild type progeny.

Cross	No. of viable progeny classified	No. of phenotypically wild-type progeny	No. of wilds tested‡	No. of PWT's	Reference¶
		Linkage Group A			
1. C94 (*bist*) × 4540 (*nic*)	3112	164	(115)	0	7, 9
2. C94 (*bist*) × 35203 (*ad-p*)	2499	99	(38) 38	0	7, 9
3. C94 (*bist*) × 15069 (*lys*)	15226	147	(68) 42	0	7, 9
4. 15300 (*al-2*)* × 34508 (*aur*)*	11815	122	(73) 20	0	9
5. 4637 (*al-1*)* × 15300 (*al-2*)*	17647	44	(19) 19	0	9
6. 4637 (*al-1*)* × 34508 (*aur*)*	ca. 5000	0	0	0	9
7. 35709 (*bmsr*) × 51504 (*bmsr*)	8792	0	0	0	3, 21
8. 15300 (*al-2*) + 3416 (*nic*) × 34508 (*aur*) + 4545 (*lys*)	15126	67	17	10	9
		Linkage Group B			
9. 3254 (*ad*) × 51602 (*ribo-t*)	10393	51	23	0	9
10. 84605 (*cys*) × 38401 (*cys-2*)	8158	39	20	4	17, 4
11. 38401 (*cys-2*) × 80702 (*cys*)	ca. 10000	0	0	0	4
		Linkage Group C			
12. 27663 (*ad*) × 10575 (*tryp*)	3624	16	16	0	9
		17†	17	13	
	Not counted	29	29	0	
		Linkage Group D			
13. 35405 (*pyro*) × 44602 (*pyro-p-2*)	5730	13	13	6	9
14. 35405 (*pyro*) × 44602 (*pyro-p-2*)	8355	25	17	9	
15. 35405 (*pyro*) × 44204 (*pyro-p-1*)†	3468	52	12	12	9
		Linkage Group E			
16. 37401 (*inos*) × 83201 (*inos-t*)	9084	6	6	0	9
	Not counted	7	7	2	
17. 37401 (*inos*) × 83201 (*inos-t*)§	Not counted	19	19	15	
18. 16117 (*iv-1*) × 39709 (*iv-3*)	15740	162	22	2	9
19. 47711 (*iv-4*)T × 39709 (*iv-3*)	5113	68	9	0	Unp., 9
		67†	8	7	
	Not counted	58	58	19	
20. 47711 (*iv-4*) × 39709 (*iv-3*)§	19168	397	48	0	

*C102, *co-t* (MITCHELL and MITCHELL 1952) in linkage group D also present in this strain.

†70007, *co*, (KÖLMARK and WESTERGAARD 1949) in linkage group D also present in this strain.

‡Subwild type progeny.

§Indicates that more than one cross was involved.

‖Figures in parenthesis in crosses 1, 2, 3, 4 and 5 indicate the number of wild types tested for bisexual strain. Figures not in parenthesis indicate number of wild types whose outcross progeny were examined.

¶Figures refer to bibliography.

strains were recovered from crosses involving mutants from the five linkage groups tested. The PWT's reported here, regardless of the mutants or the linkage groups involved, have the following characteristics: (1) They were found in the progeny of crosses between strains having closely-linked genes and were derived from individual ascospores which were normal in size. (2)

They were phenotypically wild and usually grew equally well on minimal medium or medium supplemented with the growth requirements of the parental mutant strains. (3) In crosses with standard wild-type strains the PWT's behaved as mutants since approximately 50% of the viable progeny were mutants. (4) When the parental mutant strains were phenotypically distinguishable it was possible to demonstrate that both of the parental mutants were recovered in the progeny of crosses of PWT × wild unless one of the mutant strains carried a linked translocation in which case only one of the original parental mutants was recovered. (5) All of the PWT's which have been tested proved to have mutant and wild-type macroconidia. (6) All PWT's tested have been unisexual.

Among the progeny of some of the crosses (e.g., Nos. 6, 7 and 11, table 1) neither wild nor pseudo-wild types were recovered. It is reasonable to assume that the genes involved in such crosses are alleles, although in the case of *al*-1 and *aur* different numbers of wild-type progeny were obtained in crosses with *al*-2. It is likely that there is a translocation in the *al*-1 strain which may account for this difference. Those crosses from which PWT's were recovered will be mentioned along with a more detailed description of the various PWT strains.

Linkage group E

Pseudo-wilds from inos × inos-t. Pseudo-wild types involving mutants in the E linkage group were found in the progeny of a cross involving two inositol-requiring mutants. Strain 37401 (*inos*) requires inositol for growth whereas strain 83201 (*inos-t*) requires inositol for growth only at temperatures above 35°C.

In a cross of *inos* × *inos-t* two out of the 13 phenotypically wild-type progeny which were tested segregated for inositol-requiring mutants when outcrossed to standard wild-type strains. An average of 1875 viable progeny from crosses between these two exceptional strains (14-1*A* and 14-3*a*) and standard wild-type strains were classified after 12 hours incubation at 35°C on minimal agar plates, and the percentage of mutant progeny was 47 and 53, respectively.

Mutant segregants from outcrosses involving PWT14-1*A* and PWT14-3*a* with standard wild strains were isolated and *inos* and *inos-t* were recovered from the outcross progeny of both PWT's. It was usually possible to distinguish the two inositol-requiring mutants by their growth on minimal agar plates at 35°C. On minimal medium *inos* ascospores germinate but do not grow, whereas *inos-t* ascospores germinate and usually produce several very short hyphae and often one or two aerial hyphae as well. Progeny of the cross PWT14-1*A* × wild *a* were plated on minimal agar medium and incubated at 35°C for 15 hours and 575 germinating spores were classified; 298 (52%) were wild type, 226 (39%) appeared to be *inos*, and 51 (9%) were of the type expected from *inos-t*. Under similar conditions progeny of the cross PWT14-3*a* × wild *A* were classified as follows: 381 (44%) wild type, 228 (26%) *inos*, and 261 (30%) *inos-t*.

From the cross PWT14-1*A* × wild *a* one to five asci were dissected from each of 15 different perithecia. Each ascus contained four mutant and four wild-type spores. Either *inos* or *inos-t*, but never both, were recovered from a single perithecium. Nine perithecia segregated for *inos* and six for *inos-t*. The two PWT's were also backcrossed to *inos-t* strains. PWT14-3*a* was crossed to *inos-t A* and no wild-type progeny were found in an estimated 10,000 progeny. PWT14-1*A* was crossed to *inos-t a* and among approximately 10,000 viable mutant progeny four wild types were found. Three of these wilds were checked and all were PWT's. However, it was impossible to tell whether they were PWT's which had been transmitted through the cross or whether they were simply PWT strains formed anew as a result of crossing.

In order to determine whether pure *inos* and *inos-t* strains could be recovered from these PWT's, 50 conidia from PWT14-3*a* which had germinated but grown very little on minimal agar plates after 15 hours incubation at 35°C were isolated. The growth requirement of the strains derived from these single conidia was determined and 26 strains were phenotypically wild, three were *inos* and 21 were *inos-t*. Eighteen conidia from PWT14-1*A* were tested in the same manner and 10 gave wild-type growth, one was *inos-t*, and seven were *inos*.

At 35°C neither of the two PWT strains were stimulated in their growth when inositol was added to the culture media.

Pseudo-wilds from iv-3 × iv-1. Twenty-two wild-type progeny from a cross of *iv-3 × iv-1* were outcrossed to standard wild strains and two of them segregated for mutants which required isoleucine and valine for growth. These two strains grew equally well on minimal medium or on minimal medium supplemented with isoleucine and valine. In the outcross progeny of these PWT's there was a high percentage of inviable progeny, but there were approximately 50% mutant spores among the viable progeny of the crosses.

These two mutants are indistinguishable and consequently it was impossible to determine from these experiments whether one or both of the mutants were present in the outcross progeny, but the strains were like other PWT's in that they had a wild phenotype and segregated approximately 1 : 1 for mutant and wild progeny when crossed to standard wild-type strains.

Linkage group C

Pseudo-wilds from ad × tryp. Sixteen strains from wild-type ascospores and 17 strains from subwild-type ascospores were isolated from a cross of 10575-(*tryp*) × 27663(*ad*). The 33 strains were tested in liquid cultures of minimal medium and all grew. The vials of minimal medium in which these 33 strains were growing were observed under ultra-violet light to determine whether the culture medium showed a blue fluorescence as demonstrated for the *tryp* parent due to the accumulation of anthranilic acid (TATUM, BONNER and BEADLE 1943; HASKINS and MITCHELL 1949). Thirteen of the strains, all from subwild-type ascospores, showed a blue fluorescence in the culture me-

dium. Each of the 33 strains were then outcrossed to standard wild-type strains. None of the 16 wild types or an additional 29 types tested later segregated on outcrossing, but the 13 subwild-types which showed the fluorescence all segregated for mutant progeny when crossed to standard wild-type strains.

In crosses of these PWT's to standard wild-type strains, usually three types of progeny were observed insofar as the growth of the ascospores on minimal medium was concerned. One type showed the usual wild-type growth. A second type of spore germinated but did not grow and these were invariably identified as *tryp*. A third type of spore germinated and produced a few short hyphae, and these were identified as *ad*. Both types of mutant spores were usually present when progeny of PWT × wild were plated on minimal medium; the *tryp*-type was present and the *ad*-type was not observed when spores were plated on minimal plus adenine, and the *ad*-type observed but not the *tryp*-type when progeny were plated on minimal plus tryptophane.

When the PWT's were crossed to standard wild-type strains both of the parental mutants were recovered in the progeny of 12 of the 13 PWT's. In one case involving PWT117-33 only the *tryp*-type of germinating ascospore could be found when 2000 spores were plated on minimal medium and no mutants could be found when approximately the same number of spores were plated on minimal plus tryptophane. In this cross there are several possible explanations for the failure to recover the *ad* mutant, but since they are not relevant to the main problem they will not be considered here.

From crosses of 11 different PWT's with standard wild-type strains, an average of 1126 viable progeny per cross were classified on minimal agar plates. The percentage of mutant segregants in the outcrossed progeny averaged 48 and ranged from 44 to 51.

When the 13 PWT's were grown in liquid culture of minimal, minimal + adenine, minimal + tryptophane, and minimal + adenine + tryptophane there was no significant stimulation of growth on the supplemented media. In four days growth at 25°C the 13 PWT's averaged 99 mg dry weight when grown on minimal medium, 87 mg on minimal + tryptophane, 90 mg on minimal + adenine, and 96 mg on minimal + adenine + tryptophane.

Linkage group B

Pseudo-wilds from cys × cys-2. Twenty wild-type progeny were isolated from the cross 84605 (*cys*) × 38401 (*cys-2*) and outcrossed to standard wild-type strains. Four of these strains which grew equally well on minimal or supplemented media segregated for cystine-requiring mutants. In the progeny from which the mutant segregants were recovered, the viability was low and sometimes less than 50 percent. An average of 862 viable spores were classified from the outcrossed progeny of each of the four PWT's and the percentage of mutant segregants ranged from 46 to 50.

The original parental strains of *cys* and *cys-2* were easily distinguished by their growth on various concentrations of thiosulfate. In 20 ml of minimal medium to which 500 micrograms of thiosulfate had been addeds *cys* showed

only a trace of growth whereas *cys-2* showed as much as 60 mg of growth after four days at 25°C. The original isolate of *cys* had a double requirement for both cystine and tyrosine (SHEN 1950), but the isolate which was used in this study had lost its requirement for tyrosine in the process of subculturing. In order to determine whether or not both mutants were recovered from the progeny of crosses of PWT × wild, mutant progeny from outcrosses of PWT137-12*A* and PWT137-14*A* were isolated and their growth requirements were determined. From both crosses mutant progeny were found which would not grow in minimal medium supplemented with 500 micrograms of thiosulfate and other mutants which would grow with this concentration of thiosulfate. This evidence suggests that both of the parental mutants were present in the outcross progeny, but since the growth requirements of the two mutants are so similar one could not say definitely that both of the original mutants were recovered.

Conidia from two of the four PWT's were plated on minimal medium and conidia which had germinated but had grown very little were isolated and tested. Strains derived from single conidia were recovered from both PWT's and some of these strains did not grow on minimal medium but did grow on medium supplemented with thiosulfate.

In this experiment it was not possible to show definitely that both of the original parental mutants were recovered from crosses of PWT × wild. However the PWT strains had a wild phenotype, mutant and wild type conidia were recovered from these strains and approximately 50 percent of the viable progeny from crosses with standard wild-type strains were phenotypically mutant.

Linkage group D

Pseudo-wild types involving three pyrimidine mutants in the D linkage group have been previously reported (MITCHELL, PITTENGER and MITCHELL 1952) but since that time PWT's have been found by using other mutant strains in this same linkage group and they will be mentioned briefly. Several PWT's were found among the progeny of crosses involving three pyridoxine mutants, 35405(*pyro*), 44602(*pyro-p-2*), and 44204 (*pyro-p-1*). *Pyro* requires vitamin B_6 for growth, but *pyro-p-1* and *pyro-p-2* can grow in the absence of B_6 if the pH of the culture medium is above 6 and if ammonium ions are present (STRAUSS 1951).

Pseudo-wilds from pyro × pyro-p-1, co. Twelve phenotypically wild-type progeny were selected from a cross between *pyro* and a double mutant of *pyro-p-1* and *co*, a closely linked colonial mutant. These 12 strains were outcrossed to standard wild types and the progeny of all of the crosses contained mutant segregants. Five of these strains failed to form normal perithecia the first time crosses were made, but after the strains were subcultured and again crossed, fertile perithecia were formed. The percentage of germination in these crosses was invariably low but among the viable progeny the frequency of mutants was roughly 50 percent. Five of the more fertile crosses were studied

more thoroughly than the others and both *pyro* and *pyro-p-1, co* were found among the mutant segregants of these crosses. The percentage of mutant segregants in these five crosses ranged from 47 to 52 in populations averaging 478 viable spores.

Eleven of these PWT's were grown on nitrate medium (pH 5.5), minimal medium (pH 5.5), minimal medium (pH 6.9), and minimal medium + vitamin B_6 (pH 5.5) and the average dry weights after four days growth at 25°C were 48 mg, 66 mg, 64 mg and 69 mg, respectively.

Pseudo-wilds from pyro × pyro-p-2. Pseudo-wild types were also found among the progeny of a cross between a second pH-sensitive mutant (*pyro-p-1*) and *pyro*. From two crosses between different isolates of these mutants a total of 15 PWT's were isolated. In crosses with standard wild strains the percentage of mutant progeny was approximately 50 percent, but the percent germination was very low. However, both *pyro-p-1* and *pyro* were recovered in the outcross progeny of all of the PWT's. There was no appreciable difference in the amount of growth of these PWT's on minimal medium and on minimal medium supplemented with vitamin B_6.

Linkage group A

If PWT's were found in the A linkage group which were heterozygous for the mating type alleles, it seemed logical to assume that such strains would cross with standard wild strains of both mating types and possibly be self-fertile. LINDEGREN (1934) reported isolating four strains from a single exceptional ascus which were heterocaryotic, self-fertile, and which crossed with strains of both mating types. More recently GROSS (1952) has reported heterocaryons between strains of the opposite mating types which crossed with strains of both mating types and were also self-fertile. Therefore, in the search for PWT strains involving the mating type chromosome, attempts were first made to find wild-type progeny from crosses of linked mutants which would cross with strains of both mating types. All such wild-type strains were also grown alone on crossing medium to check for self-fertility.

From the first five crosses (table 1), involving mutants on the mating type chromosome, a total of 313 wild-type progeny were isolated. Of these strains 298 crossed with either one mating type or the other but never both, and 15 would not cross with strains of either mating type. The outcross progeny of 119 of these strains were examined and in only one case did parental mutants segregate in the outcross progeny. The one phenotypically wild-type strain which segregated for both of the original parent mutants when crossed to a standard wild-type strain and from which mutant conidia could be isolated was unisexual and came from a cross of *al-2, co-t* × *al-1, co-t*. This strain had all of the characteristics of a PWT except that it could not definitely be established as having arisen from a single ascospore since it was isolated as a colony, as described earlier. Consequently, it was not classified as a PWT.

Pseudo-wilds from al-2, nic, a × aur, lys, A. Four linked mutants, *al-2*, *aur*, *lys* and *nic*, which are in the opposite arm of the chromosome from the mating

type locus, were used in a cross from which ten different PWT's were recovered. Random spore counts (see table 1) from a cross between *al-2* and *aur* (a deviate pigment type probably an allele of *al*-1, HUNGATE 1945) indicate that these two genes are approximately two units apart. Although *aur* strains usually have a mixture of albino and pigmented conidia at maturity it is not always possible to distinguish phenotypically all isolates of *al-2* and *aur*. Two different crosses between *lys* and *nic* gave an average of 0.44% wild-type progeny in a total of 15,126 viable spores indicating that these genes are slightly less than one unit apart. *Lys* is distal to *al-2* (DOERMANN 1946) but the actual distance between these two mutants has not been accurately determined.

From the progeny of a cross between *al-2, nic, a* and *aur, lys, A* 57 wild-type and subwild-type germinating ascospores were isolated from among several thousand mutant progeny plated on minimal medium. At maturity 40 of these isolates were albino and all appeared to be *al-2* rather than *aur*. The other 17 isolates were non-albino. Since PWT's involving *al-2, nic, aur* and *lys* as well as certain multiple crossovers would be expected to be phenotypically wild, only the 17 non-albino wild types were saved for further study. A dilute suspension of conidia from each of these 17 strains was plated on both minimal medium and medium supplemented with lysine and niacin. The medium also contained 1% sorbose and 0.2% sucrose to induce colonial type growth. After a period of four days at 30°C the plates were examined and on the 17 plates of minimal medium and on seven of the supplemented plates only non-albino colonies were observed. However, on ten of the plates supplemented with lysine and niacin both albino and non-albino colonies were observed. Conidia from the ten exceptional strains were then plated on sorbose medium + lysine and sorbose medium + niacin. Albino and non-albino colonies were observed on all of the plates. Several albino colonies were isolated from each plate and their growth requirements checked again in liquid cultures of minimal and supplemented media. These tests confirmed that from each of the ten phenotypically wild-type strains both niacin- and lysine-requiring albino strains could be isolated from single conidia. It was assumed that the albino niacin-requiring strains were *al-2* and that the albino lysine-requiring strains were *aur*, although the *aur* and *al-2* isolates from a single PWT were phenotypically different in only four of the ten cases. However both *al-2* and *aur* must have been present in order to account for the pigmentation of the conidia of the PWT strains.

The ten PWT's were outcrossed to standard wild-type strains and four of them were mating type *a* and six were *A*. Both lysine- and niacin-requiring segregants were recovered from the outcrossed progeny of each of the ten PWT strains. Four of the seven non-albino wild-types which did not segregate for albino colonies when conidia were plated on supplemented medium were outcrossed and only wild-type progeny were recovered. Three of the ten PWT strains formed perithecia when grown alone on crossing medium and when crossed to either mating type. However, these strains formed fertile perithecia

with only one mating type; the perithecia formed when grown alone or in crosses with the other mating type were sterile. As a further check on mating type the niacin- and the lysine-requiring strains isolated from conidia of each of the ten PWT's were tested separately for mating type and both mutant components of each PWT had the same mating type reaction. Thus it was concluded that each of the ten PWT's were homocaryotic for mating type but heterocaryotic for *al-2, aur, lys* and *nic*.

All of the PWT's came from ascospores which were scored as subwild when isolated from the plates of minimal medium, but all subwild type ascospores were not PWT's. Progeny of three of the more fertile crosses of PWT × wild were studied to determine the frequency of mutant and wild types. An average of 800 viable spores per cross were classified and the percentage of mutant progeny ranged from 48 to 52.

Pseudo-wild types involving a reciprocal translocation

PWT strains have previously been described (MITCHELL et al. 1952) which were recovered from a cross of two mutant strains one of which carried a reciprocal translocation linked to the mutants. These PWT's were different from others which have been described in that either one or the other, but never both, of the parental mutants was recovered when any one PWT was crossed to a standard wild strain. Since that time PWT's have been found in a cross of *iv*-4*a* × *iv*-3*A* (both genes are located in the E linkage group) and the genetic evidence indicates that strain *iv*-4*a* carries a reciprocal translocation although no attempt has yet been made to identify the translocation cytologically. PWT's from this cross were studied not only to determine whether or not they were similar to those previously described which involved a translocation but also because they are important from the point of view of the origin of PWT strains.

The presence of the translocation in strain *iv*-4*a* was first suspected when it was found that the spore abortion patterns in crosses involving this strain were similar to those described by MCCLINTOCK (1945) and SINGLETON (1948) in crosses involving known translocations. In the progeny of the cross *iv*-4*a* × *iv*-3*A* it was found that 42 out of 49 wild types scored as recombinants were mating type *a* and also showed spore abortion patterns in the asci in crosses to standard wild-type strains suggesting that the A linkage group along with the E linkage group was involved in the reciprocal translocation. Additional evidence also indicated that the A linkage group was involved. In a cross of *iv*-4*a* with C140*A*, a histidine requiring mutant in the A linkage group (HAAS et al. 1952), 18 complete asci were checked and only one ascus showed any recombination between the two mutants. The observation of pseudo-linkage of *iv*-4 and C140 in asci, while random spore counts from the same cross showed little or no evidence of linkage, strongly suggested the presence of a translocation involving the A and E linkage group. Close linkage of mating type *a* and the translocation and loose linkage of *iv*-4 and the translocation was suggested by the fact that out of a total of 46 randomly selected *iv*-mutant strains from

the progeny of the cross of *iv*-4*a* × wild *A*, 27 were *a* and all but one carried the translocation judging from the spore abortion patterns in the asci; the other 19 strains were *A* and none of these showed evidence of the translocation.

Among 5113 viable spores from the cross of *iv*-4*a* × *iv*-3*A*, 97.4 percent were mutant, 1.3 percent were wild, and 1.3 percent were subwild or intermediate between mutant and wild-type growth. Nine wild types were tested and all were recombinants, whereas seven of the eight subwilds which were tested were PWT's. In all 26 PWT's were isolated from this cross. After crossing to standard wild-type strains it was found that the PWT's could be divided into three classes on the basis of mating type (*A* or *a*) and the presence or absence (T or +) of the translocation which was detected by the spore abortion patterns within the asci. The frequency and phenotype of the 26 PWT's were as follows: 12 *a*T (Class I), 13 *A*+ (Class II) and one *a*+ (Class III).

All of the PWT's mentioned here were similar to others which have been described in that approximately 50 percent of the progeny in crosses with standard wild-type strains were mutants. Macroconidia from a Class I PWT(13-13*a*) and a Class II PWT(13-21*A*) were plated on minimal agar and 99 and 88 conidia which had germinated but showed very little evidence of growth were isolated from the respective cultures. In both cases 11 percent of the strains derived from these conidia required isoleucine and valine for growth. Twenty-six asci from the cross of a Class III PWT × wild *A* were dissected and the growth requirement of the spore pairs was determined. Each ascus contained four wild and four *iv*-mutant spores. All of the PWT's grew in liquid cultures of minimal medium when they were first isolated which confirmed their wild phenotype. On the average Class I PWT's showed less growth on minimal medium than did Class II PWT's. However, there was no consistent difference in the dry weight of any of the PWT's when grown on supplemented medium as compared to minimal medium. Class I PWT's differed from Classes II and III in that after being cultured three to four times on minimal medium and then stored at low temperatures for several months, seven out of the twelve PWT's failed to grow or grew very little on minimal medium. On the other hand, Classes II and III grew as well on minimal medium after subculture and storage as they had originally.

The most logical explanation of the origin of the three different classes of PWT's is that they arose as a result of 3–1 segregation of the ring-of-four chromosomes at anaphase. It was assumed that the two chromosomes carrying the *iv*-mutants must have undergone adjacent-2-segregation (chromosomes with homologous centromeres going to the same pole) in order for the PWT's to have a wild phenotype and also to segregate for *iv*-mutant progeny. When these two chromosomes underwent adjacent-2-segregation the other two chromosomes in the ring (those carrying the mating type alleles) must have segregated at random since 13 PWT's were *a* and 13 were *A*. Although it is impossible at the present time to distinguish *iv*-3 and *iv*-4 from one another it seems likely by analogy with a previously studied example (MITCHELL et al. 1952) that only *iv*-4 is recovered from Class I PWT's because of the *a* genotype and

the segregation of the translocation, whereas only *iv*-3 is recovered from Class II PWT's which are *A* and do not segregate for the translocation.

In crosses of *iv*-4*A* × *iv*-3*a*, in the absence of the translocation, there were 2.1 percent wild-type progeny in a total population of 19,168 viable spores. Forty-eight wild types were tested and no PWT's were found. This suggests that the percentage of PWT's among the viable progeny is much higher when one of the parental mutant strains carries a linked translocation.

Evidence that pseudo-wilds are not derived from ascospores with two genetically different nuclei

The fact that pure mutant strains could be isolated from single conidia from PWT strains definitely establishes the fact that they were heterocaryotic, at least during the later stages of their growth. There was no direct evidence, however, to indicate whether PWT's were heterocaryons because they were derived from heterocaryotic ascospores, or whether they originated as disomic strains which underwent some sort of somatic reduction giving rise to a heterocaryon made up of haploid and disomic nuclei. To get additional evidence on this point the following experiment was carried out. This experiment was based on the fact that a heterocaryotic strain arising from an ascospore having a disomic nucleus would be heterocaryotic with respect to markers on only one chromosome pair, whereas if the strain originated from a heterocaryotic spore it would be possible for it to be heterocaryotic for markers on more than one of the chromosomes.

Crosses were made between a double mutant *inos-t, co A* and four different isolates of *inos a*. *Inos* and *inos-t* are located in the E linkage group and *co* is in the D linkage group. Ascospores of the genotype *co* germinate and grow in such a way that they are easily distinguished from non-colonial strains. Heterocaryons involving *co* have been repeatedly observed in other experiments. Nineteen inositol-independent strains were selected from the progeny of these crosses and outcrossed to standard wild-type strains. None of them crossed to both mating types. Fifteen of the wild strains segregated for inositol mutants in the outcross progeny and four did not. The phenotype of these 19 inositol-independent strains was as follows:

Wild types			Pseudo-wild types		
Mating type	Growth habit	No. recovered	Mating type	Growth habit	No. recovered
A	Colonial	1	a	Wild	4
A	Wild	2	A	Wild	7
a	Colonial	1	a	Colonial	1
		4	A	Colonial	3
					15

Two to three thousand germinating ascospores from the outcross progeny of each of the 11 non-colonial PWT's were examined on minimal agar plates supplemented with inositol and no colonial types were found. Both *inos* and *inos-t* were recovered from the outcross progeny of each of the 11 PWT's.

Approximately 10,000 conidia from each PWT were plated on inositol-medium and examined after they had grown enough so that colonial and non-colonial segregants could be expected to be distinguished. There was no evidence of colonial growth when conidia of any of the 11 PWT's were tested. The recovery of both of the inositol mutants and the failure to find any evidence of the colonial mutant indicates that the non-colonial PWT's were pure with respect to the normal allele of the colonial gene. The four colonial PWT's were also checked and when outcrossed to standard wild strains approximately 50 percent of the progeny were colonial and both *inos* and *inos-t* segregants were recovered. Germinating conidia from these strains were also examined and no non-colonial types were observed. The failure to obtain strains heterocaryotic for *co* strongly indicates that PWT's do not arise from heterocaryotic ascospores.

DISCUSSION

The recovery of pseudo-wild type strains involving mutants from the five linkage groups so far studied clearly demonstrates that PWT's are associated not only with the D linkage group where they were first found, but also with each of the other linkage groups tested. The isolation of 99 different pseudo-wild strains indicates that the phenomenon of PWT's is one of widespread occurrence in the various linkage groups and emphasizes the necessity, as previously pointed out by MITCHELL et al. (1952) and recognized by GILES and PARTRIDGE (1953) of considering PWT's when attempting to evaluate genetic data from crosses involving closely linked or possibly allelic genes in Neurospora. If nondisjunction is the phenomenon giving rise to PWT's then it may deserve consideration in genetic studies in other haploid organisms as well.

It has previously been pointed out that the origin of PWT strains is consistent with the view that they are derived from nuclei with $n + 1$ chromosomes if one assumes that the disomic nuclei are unstable in mitotic divisions and give rise to heterocaryons containing both disomic and haploid nuclei and that only haploid nuclei function in fertilization. PWT strains, whether originating as disomics or as heterocaryotic ascospores, might be expected to have a wild phenotype, to behave as mutants in crosses and to have mutant and wild-type macroconidia. However, in addition to the reasons previously presented (MITCHELL et al. 1952) for believing that PWT strains did not originate as heterocaryotic ascospores, the failure to detect in a heterocaryotic condition a marker gene, for example *co*, in a linkage group independent of the one carrying the genes in the PWT strongly suggests that PWT's do not arise from heterocaryotic ascospores.

In the search for PWT's involving the A linkage group, the failure to find any evidence of PWT's in three different crosses of mutants closely linked to the mating type locus initially suggested that the phenomenon giving rise to such strains in this chromosome was rare. However pseudo-wild strains were recovered in a later experiment from the progeny of a cross involving four

mutants (*al-2, aur, lys* and *nic*) which were not closely linked to the mating type locus. All of these PWT's were homocaryotic for either one or the other of the mating type alleles but heterocaryotic for the four genes on the opposite arm of the chromosome. This failure to recover bisexual PWT's suggests a definite selection against strains carrying both of the mating type alleles or possibly some other genes closely linked to this locus. Bisexual PWT's are probably initially formed but whether they are inviable or whether they might simply have had such a slow growth rate similar to bisexual heterocaryons (BEADLE and COONRADT 1944; GROSS 1952) that they were not recognized as wild types in the initial selection process is unknown.

It is of interest to note the low frequency of both wild and pseudo-wild types in the progeny of several of the crosses between mutant strains having similar or identical phenotypes. The available evidence suggests that *inos* and *inos-t*, *cys* and *cys-2*, and *pyro* and *pyro-p* are all less than one unit apart although the large number of inviable progeny in some of the crosses makes it difficult to determine accurately the distances separating these genes. What we may call true wild types as compared to pseudo-wild types in the progeny of crosses between members of any one of the three groups of mutants just mentioned may represent crossovers between pseudo alleles. Further study, however, using closely linked marker genes would be needed to establish the nature of such true wild types. The isoleucine-valine mutants as well as the albino mutants are also closely linked but they seem to be too far apart to be typical examples of pseudoalleles.

Although a thorough investigation of the allelic relationship between several pairs of genes used in these experiments has not been attempted, the recovery of both wild and pseudo-wild types in the progeny of crosses between strains having similar or identical phenotypes strongly suggests that two genes with different functions are involved. However one cannot rule out the possibility that in rare instances a dosage effect of two allelic genes might give PWT's. The use of PWT's is equivalent to the heterocaryon method of demonstrating non-allelism. Moreover, the production of PWT's should be a useful method of obtaining heterocaryons in cases where they cannot be obtained by standard methods.

SUMMARY

Pseudo-wild type strains which are phenotypically wild but behave as mutants in crosses have previously been found in the progeny of crosses between mutants located in the D linkage group of *Neurospora crassa* (MITCHELL, PITTENGER and MITCHELL 1952). In the studies reported here the progeny of 20 crosses involving closely linked or possibly allelic genes in five different linkage groups were analyzed for the presence of PWT strains. Wild-type progeny were observed in 17 of the 20 crosses, but PWT's were found in only 10 of these. In all, 654 phenotypically wild-type progeny from these crosses were analyzed and 99 different PWT strains were found. PWT's involved mutants in each of the five linkage groups tested. PWT strains were

recovered involving mutants in the chromosome bearing the mating type locus, but these PWT's were invariably unisexual. This suggests a selection against PWT strains having both mating type alleles. The recovery of 99 different PWT's indicates that the phenomenon giving rise to such strains is one of widespread occurrence in the various linkage groups and should be given careful consideration in evaluating genetic data involving closely linked or possibly allelic genes.

PWT strains, regardless of the mutants or the linkage group involved, have the following characteristics: (1) They are derived from normal-sized ascospores. (2) They are phenotypically wild and grow equally well on minimal medium or medium supplemented with the growth requirements of the parental mutant strains. (3) In crosses of PWT's with standard wild-type strains approximately 50 percent of the progeny are mutant. (4) When PWT's are outcrossed to standard wild strains it is possible to demonstrate that both of the original parental mutants, if they are phenotypically distinguishable, are recovered in the outcross progeny unless one of the parental mutant strains carries a reciprocal translocation, in which case only one of the parental mutants is recovered from any one PWT. (5) Mutant and wild-type macroconidia are recovered from all of the PWT strains which have been tested. (6) All PWT's tested have been unisexual. In one group of crosses from which PWT's were recovered the two linked mutants involved in the PWT's were always heterocaryotic, whereas an unlinked colonial marker gene in non-colonial PWT's was never found in a heterocaryotic condition. The phenotype and behavior of a large number of PWT's is consistent with the suggested explanation of their origin from disomic nuclei.

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GENETIC ANALYSIS OF POLYPLOID YEAST[1]

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THE yeast *Saccharomyces cerevisiae* normally alternates between haplophase and diplophase, propagating vegetatively primarily as a diploid (WINGE 1935). SUBRAMANIAM and coworkers (SUBRAMANIAM and KRISHNA MURTHY 1949; MITRA and SUBRAMANIAM 1949; SUBRAMANIAM 1951) have reported the production of tetraploids by the exposure of diploid yeast to acenaphthene and chrysene. Their data lack any genetic support. ROMAN, HAWTHORNE and DOUGLAS (1951) have described a single aberrant ascus, which they suggest might have arisen from a tetraploid. A genetic analysis of tetraploids produced by mass mating of diploid gametes (LINDEGREN and LINDEGREN 1951) was obscured by uncontrolled sporulation of the diploids arising from the tetraploids. These workers also report the production of a triploid, but present no genetic data for this culture.

The isolation of triploid and tetraploid clones by means of a prototroph recovery procedure has been reported (POMPER 1952a, b). This paper will describe some of the results of genetic analyses carried out on triploid and tetraploid clones of *S. cerevisiae*.

MATERIALS AND METHODS

The strain of *S. cerevisiae* in which biochemically altered mutants were produced (POMPER and BURKHOLDER 1949) is heterothallic, mating types being designated *a* and *α* (LINDEGREN and LINDEGREN 1943). Normally, haploids of the same mating type will not conjugate with one another. A diploid, heterozygous for mating type, will yield no observable conjugation figures if mixed with haploids or other diploids.

To permit the isolation of polyploids from crosses of cultures that would normally be considered nonmating, the prototroph recovery procedure (LEDERBERG and TATUM 1946), as adapted for yeast (POMPER and BURKHOLDER 1949), was employed. In a cross of haploids ($a \times \alpha$), the yield of prototrophic diploids after 3 days incubation is sufficiently large for a 3-mm loopful of washed-cell suspension streaked on minimal agar to produce heavy growth. For isolation of polyploids, however, it was necessary to plate more than 10^6 cells in order to recover any prototrophs; and some attempted crosses yielded no detectable prototrophs. Strains requiring two biochemical factors were

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usually employed in these experiments to minimize the probability of recovering back mutants. The gene-controlled biochemical deficiencies were for tryptophan (*Tr*), methionine (*Me*), adenine (*Ad*), and uracil (*Ur*).

Genetic analysis was routinely run as follows: The prototrophic isolate was inoculated onto presporulation agar (LINDEGREN and LINDEGREN 1944) and incubated at 25°C for 2 to 3 days. Cells were then scraped off and spread on an agar slant composed of 0.02 percent raffinose, 0.22 percent sodium acetate, and 2 percent agar (after ADAMS 1949). Sporulation occurred on these slants at 25°C in 2 to 7 days. After dissection, the ascospores were germinated in hanging drops, then transferred successively to broth and complete agar stock

TABLE 1

Analysis of segregants from triploids.

Ascospore*	M.T.	*Tr*	*Me*	*Ad*	*Ur*	Sporulation
	Cross: $a Tr^- Me^- \times a/\alpha\ Tr^+/Tr^- Me^+/Me^- Ad^-/Ad^- Ur^-/Ur^-$					
A541.1	−	+	−	+	+	+
A541.2	*a*	−	+	+	−	−
A541.3	α	−	+	−	+	−
A541.4	*a*	+	−	+	+	−
A544.1	*a*	−	−	+	+	−
A544.2	*a*	+	+	+	+	−
A544.3	*a*?	−	−	−	+	−?
A544.4	−	+	+	+	+	+
A555.1	*a*	+	+	+	+	−
A555.2	*a*	+	−	+	+	−
A555.3	−	−	+	+	−	+
A555.4	−	−	−	+	+	+
	Cross: $\alpha Tr^- Me^- \times a/\alpha\ Tr^+/Tr^- Me^+/Me^- Ad^-/Ad^- Ur^-/Ur^-$					
A547.1	−	+	+	+	+	+
A547.2	α?	+	−	+	+	−?
A547.3	α	−	+	+	+	−
A547.4	α	−	−	+	+	−
A552.1	*a*?	+	+	+	+	−
A552.2	α	−	−	+	+	−
A552.3	*a*?	+	+	+	+	−
A552.4	α	−	−	−	+	−
A554.1	α	−	+	+	+	−
A554.2	−	+	−	+	+	+
A554.3	*a*?	−	−	−	+	−
A554.4	α	+	+	+	+	−

M.T. = Mating type. Other symbols are given in text.
+ = Independent of requirement.
− = Dependent upon factor for growth.
* = Figures preceding the period indicate ascus number; following the point, figures indicate spore number.

Mating type is listed as "−" if no conjugation-like figures were observed. In some cases, figures were observed which were difficult to judge, and these are listed as "?". Prototroph recovery test crosses were used to further this analysis where possible. A552.1 and A552.3 could not be thus tested because of their nutritional independence; the other questionable cases were all negative on plating, indicating that they were probably *a*/α.

slants. Analyses of mating types and nutritional requirements were carried out either on cells taken directly from stock slants, or from slants inoculated on the previous day.

Mating type is scored by mixing loopfuls of unknown with known tester stocks of both mating types, and observing under the microscope 2 to 3 days after placing at 25°C. Cultures are scored as "+" only if there is an abundance of characteristic conjugation figures. In questionable cases, as in table 1, prototroph recovery with suitable tester stocks is used as a final proof of mating type status (POMPER and BURKHOLDER 1949). Analysis of the nutritional requirements is done by single omission tube tests: A needlepoint of cells is taken from a slant, suspended in 1 to 2 ml of sterile water, and one drop of this suspension used per tube. The tubes are rotated continuously at 30°C for 1 to 3 days. Cultures are scored simply as "+" or "−". In doubtful cases, quantitative growth measurements are made (POMPER 1952c), and/or population analyses run.

Irradiation procedures will be described in detail elsewhere. The source of X-radiation was a G.E. Maxitron 250 unit, operated at 250 kv peak, 30 ma, 3 mm aluminum filtration added, intensity ca. 1800 r per minute.

RESULTS AND CONCLUSIONS

Triploids

A diploid of genotype $a/\alpha Tr^+/Tr^-Me^+/Me^-Ad^-/Ad^-Ur^-/Ur^-$ was crossed with *a* and α haploids of $Tr^-Me^-Ad^+Ur^+$ genotype, prototrophs isolated and induced to sporulate. Relatively good germination of the ascospores was obtained. In the cross $a/\alpha \times a$, twenty of twenty-four spores germinated; in the cross $a/\alpha \times \alpha$, twenty-nine of forty-two germinated. Three asci from each cross yielded four surviving spores. The genetic analyses of these asci are shown in table 1. The terms "spore" or "ascospore" will be used in this paper to designate the culture derived from the original dissected spore.

Three facts illustrated in table 1 should be noted: (1) Six of the 24 spores do not mate at all, and of the five scored as questionable, three were shown

TABLE 2

Genotypes of ascospores from two diploids derived from a triploid.

Ascospores	M.T.	*Tr*	*Me*	*Ad*	*Ur*
A555.3 (Phenotype: M.T.−, $Tr^-Me^+Ad^+Ur^-$)					
A567.1	*a*	−	−	−	−
A567.2	*a*	−	+	+	−
A567.3	α	−	+	−	−
A567.4	Died				
A555.4 (Phenotype: M.T.−, $Tr^-Me^-Ad^+Ur^+$)					
A565.1	α	−	−	−	−
A565.2	*a*	−	−	+	+
A565.3	*a*	−	−	−	−
A565.4	α	−	−	+	+

TABLE 3

Analysis of cross between A555.2 (from triploid) and a standard haploid.

Ascospores	M.T.	*Tr*	*Me*	*Ad*	*Ur*
Parental genotypes: A555.2 ($aTr^{+}Me^{-}Ad^{+}Ur^{+}$) × A188.2 ($\alpha\, Tr^{-}Me^{+}Ad^{-}Ur^{-}$)					
A683.1	*a*	−	+	−	+
A683.2	*a*	−	−	+	−
A683.3	α	+	−	+	−
A683.4	α	+	+	−	+

not to mate by prototroph recovery tests; (2) the phenotypic segregations for genes *Tr* and *Me* are 2+ : 2− in all six asci, and for those for *Ad* and *Ur* are consistently 3+ : 1− or 4+ : 0−; and (3) the six nonmating ascospores themselves sporulated. It may be noted further that two of the four spores in ascus A555 sporulated, and so this ascus was selected for further analysis.

Table 2 summarizes the analysis of two asci produced from A555.3 and A555.4. It is clear that these spores were initially heterozygous for mating type, which explains their failure to conjugate with tester stocks. Thus, from the direct analysis of the sporulating ascospores A555.3 and A555.4, it is

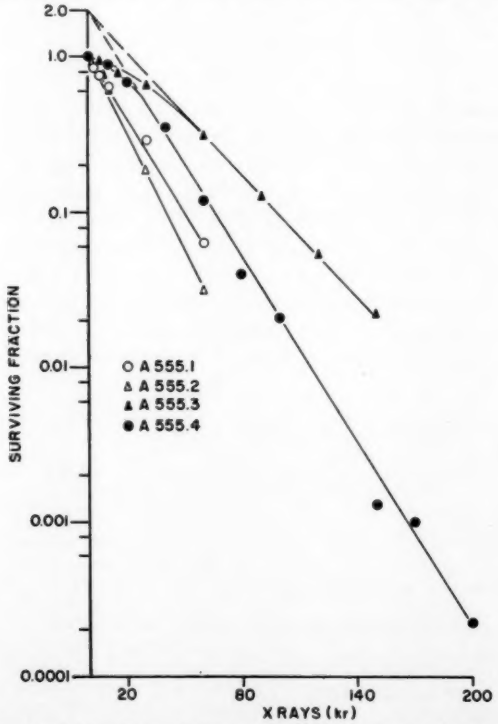

FIGURE 1.—Survival after X-irradiation of four spores from a triploid ascus (A555).

clear that both are true diploids. When ascospore A555.2 was mated with a known haploid, the segregations for mating type and the four biochemical marker genes were 2:2 (table 3), indicating the haploid nature of A555.2. Ascospore A555.1 (independent for the four nutritional requirements) was not thus tested because a prototrophic diploid could not be recovered from it.

As an adjunct to the genetic analysis of ascus A555, a series of X-ray-inactivation experiments were carried out. It was hoped that further insight into the ploidy of the cultures might thus be obtained (ATWOOD and NORMAN 1949). Figure 1 shows the survival curves of the four cultures. A555.1 and A555.2 were killed exponentially, as might be anticipated for haploids (LATARJET and EPHRUSSI 1949; NORMAN 1951; POMPER and ATWOOD in press). Cultures A555.3 and A555.4 gave sigmoidal survival curves, extrapolating to approximately 2 at zero dose.

Thus the genetic and radiokinetic data strongly suggest that in this ascus (A555) the initial segregation is into two diploids and two haploids. The data in table 1, showing that some spores in other asci also sporulate, suggest that

TABLE 4

Genetic analysis of a triploid ascus.

Ascospores	M.T.	*Tr*	*Me*	*Ad*	*Ur*
Cross: $aTr^-Me^- \times a/\alpha\ Tr^+/Tr^-Me^+/Me^-/Ad^-/Ad^-Ur^-/Ur^-$					
A555.1	*a*	+	+	+	+
A555.2	*a*	+	−	+	+
A555.3	*a*/α	−/−	+/−	+/−	−/−
A555.4	*a*/α	−/−	−/−	+/−	+/−
Over-all ratios	4*a*:2α	2+:4−	2+:4−	4+:2−	3+:3−

these cultures are also diploids. Therefore, as a working hypothesis, we conclude that a triploid may segregate out two diploids and two haploids. In those cases where a culture does not sporulate, its identification as a diploid or haploid must be based on additional evidence, such as radiokinetic analysis or behavior in a cross.

Table 4 summarizes the genetic segregation of A555 in detail. A ratio of 4*a*:2α is obtained for the mating type locus, as expected from a cross of *a*/α × *a*. Ratios of 2+:4− are obtained for the *Tr* and *Me* genes, as expected from crosses of +/− × −. The ratios for *Ad* (4+:2−) and *Ur* (3+:3−) are inconsistent with the 2+:4− segregation expected from a cross of −/− × +.

To examine the effect of reversing the marker genes, crosses were carried out between haploids recessive for *Ad* and *Ur*, and a diploid homozygous recessive for *Tr* and *Me*. It seems evident (table 5), despite the small sample, that in these crosses no excess of independent types is observed. Therefore, the aberrant ratios obtained for the *Ad* and *Ur* genes in table 1 are to be regarded as a result of an as yet undefined peculiarity of the culture(s) involved.

Aberrant ratios were also obtained from another cross, $aMe^- \times a/\alpha$

$Tr^-/Tr^-Me^+/Me^-Ad^-/Ad^-Ur^-/Ur^-$. A prototrophic isolate was sporulated and dissected. Fairly good ascospore germination was obtained, although no complete asci survived. In two asci with three surviving spores per ascus, ratios of 3+ : 0– were obtained for *Tr* and *Ad*, respectively. Population analyses revealed no heterogeneity, suggesting either that mutation had occurred very early after germination, or that this culture, like those analyzed in table 1, undergoes a meiosis whose details we do not understand.

TABLE 5

Segregation of triploids whose genetic markers are reversed as compared with those analyzed in table 1.

Ascospore	M.T.	*Tr*	*Me*	*Ad*	*Ur*	Sporulation
	Cross: $aAd^-Ur^- \times a/aTr^-/Tr^-Me^-/Me^-Ad^+/Ad^-Ur^+/Ur^-$					
A579.1	*a*?	–	–	+	–	–
A579.2	*a*	+	+	–	+	–
A579.3	Died					
A579.4	*a*	+	–	+	+	–
A581.1	*a*	+	–	–	–	–
A581.2	*a*	–	+	–	–	–
A581.3	*a*	–	+	+	+	–
A581.4	Died					
A583.1	*a*	–	–	–	–	–
A583.2	–	+	+	+	+	–
A583.3	*a*	–	+	–*	–	–
A583.4	*a*	+	–	+	+	–
	Cross: $aAd^-Ur^- \times a/aTr^-/Tr^-Me^-/Me^-Ad^+/Ad^-Ur^+/Ur^-$					
A576.1	*a*?	–	+	–	–	–
A576.2	Died					
A576.3	*a*?	–	–	–	+	–
A576.4	*a*	+	+	+	–	–
A577.1	*a*	+	–	–	–	–
A577.2	*a*	–	+	+	+	–
A577.3	*a*	+	–	–	+	–
A577.4	Died					

***A583.3 characteristically grew slowly in the absence of adenine. A mixed population of dependent and independent types (with a large excess of the former) could be demonstrated by population analysis. The assumption has therefore been made that the original genotype was Ad^-.**

Eight other crosses were made, but poor ascospore germination prevented satisfactory analyses. For example, in a cross of $a/aTr^-/Tr^-Me^+/Me^-Ad^+/Ad^-Ur^-/Ur^- \times aMe^-Ad^-Ur^-$, three spores germinated from five asci dissected. In a cross of $a/aMe^-/Me^- \times aTr^-Ad^-Ur^-$, four spores survived from six asci dissected.

Tetraploids

Five presumed tetraploids have been produced by prototroph recovery. Only one ascus, from the cross A541.4 × A555.3, has, on dissection, yielded four sporulating diploid spores. In another ascus of this cross, two sporulat-

ing (and nonmating) and two nonsporulating (and mating) spores of opposite mating types were isolated. One of the mating spores (A585.2), of *a* mating type, was crossed with two known haploids of *a* mating type to determine its genetic status. From ten four-spored asci, only ten spores germinated, and no complete asci were obtained. Thus the only ascus that can be definitely analyzed is the one in which all four spores themselves sporulated. However, the inference from the crosses of A585.2 by the known haploids is that triploids were produced, and that A585.2 is probably diploid (compare with test cross of A555.2 in table 3). Clearly, further data on this point are desirable.

TABLE 6

Analysis of four diploid ascospores from a tetraploid (A541.4 × A555.3).

Ascospore	M.T.	*Tr*	*Me*	*Ad*	*Ur*
	A588.1 (Phenotype: M.T.−, $Tr^-Me^-Ad^+Ur^+$)				
A591.1*	*a*	−	−	+	+
A591.2	α	−	−	+	+
A591.3	α	−	−	−	+
	A588.2 (Phenotype: M.T.−, $Tr^+Me^-Ad^+Ur^-$)				
A596.1*	*a*	+	−	+	−
A596.2	α	−	−	+	−
A596.3	α	−	−	+	−
	A588.3 (Phenotype: M.T.−, $Tr^-Me^+Ad^-Ur^-$)				
A601.1*	α?	−	+	−	−
A601.2	*a*	−	+	−	−
A601.3	α	−	−	−	−
	A588.4 (Phenotype: M.T.−, $Tr^+Me^+Ad^+Ur^+$)				
A606.1	α	−	−	−	+
A606.2	*a*	−	+	+	+
A606.3	α	+	+	+	+
A606.4	*a*	+	−	−	+
Over-all ratios	4*a*:4α	2+:6−	2+:6−	4+:4−	4+:4−

*Three-spored asci analyzed because four-spored asci were not found.

Inasmuch as four sporulating spores were obtained from the cross A541.4 × A555.3, it seems most economical to conclude that the prototrophic isolate was indeed tetraploid. It should be noted that culture A541.4 (see table 1) did not sporulate, and must be described as an " inferred " diploid. Since it mated as an *a* type and required methionine, it was assumed to be $a/aMe^-/Me^-$. Culture A555.3 (see table 4) was $a/aTr^-/Tr^-Me^+/Me^-Ad^+/Ad^-Ur^-/Ur^-$. The segregation data for the four sporulating spores of ascus A588 from this cross (table 6) may be used to elucidate the genotype of the incompletely analyzed parent A541.4. The observed ratios lead to the conclusion that A541.4 was $Tr^+/Tr^-Me^-/Me^-Ad^+/Ad^-Ur^+/Ur^+$. It should be emphasized that these segregation data do not prove that the prototrophic isolate A541.4 × A555.3 is tetraploid, because of the " inferred " diploid status of

A541.4. However, the occurrence of four sporulating spores in a single ascus is certainly consistent with the view that both parents were diploids and that the isolate was tetraploid. It should further be noted that the over-all segregation for mating type in table 6 is anomalous. Instead of the expected $6a:2a$, a ratio of $4a:4a$ was observed. Since mutation of mating type in haploids has been demonstrated (POMPER and MCKEE 1953), it is possible that a cell of A555.3 mutated from a/a to a/a prior to the cross, thus giving rise to the $4a:4a$ ratio.

There are at least two indications that isolates presumed to be tetraploids from prototroph isolation may undergo peculiar meioses. From a cross of two a/a diploids, four asci were obtained in each of which three spores survived. One might expect non-mating (a/a) diploids to be a frequent class (50 percent) in the meiotic products. The observed results from this cross

TABLE 7

Analysis of seven-spored and four-spored asci from tetraploid (A585.2 × A588.1).

Ascospore	M.T.	*Tr*	*Me*	*Ad*	*Ur*
Cross: $a/a\ Tr^+/Tr^-\ Me^+/Me^-\ Ad^-/Ad^-\ Ur^-/Ur^- \times a/a\ Tr^-/Tr^-\ Me^-/Me^-\ Ad^+/Ad^-\ Ur^+/Ur^+$					
A629.1	*a*	+	−	+	+
A629.2	−	−	+	+	+
A629.3	−	+	+	+	+
A629.4	*a*	+	−	−	+
A629.5	*a*	−	+	−	−
A629.6	*a*	−	−	−	+
A629.7	Died				
A631.1	*a*	+	−	+	+
A631.2	*a*	−	+	−	+
A631.3	*a*	−	+	+	+
A631.4	*a*	+	−	−	+

The genotype of the a/a parent is inferred since it did not sporulate. It was an isolate from the tetraploid analyzed in table 6, and the inferences were made in accordance with the data summarized there.

were that five of the twelve ascospores mated with *a* stocks, and seven mated with *a* stocks. If these isolates were all diploids—and their microscopic appearance suggested that they might be—it is indeed remarkable that no non-mating progeny were recovered.

The second datum suggesting a peculiar meiosis comes from a cross of A585.2 × A588.1. Ascospore A585.2 was isolated from the cross A541.4 × A555.3. Its genotype was inferred, since it did not sporulate. Ascospore A588.1 was analyzed in table 6. An ascus containing seven spores, instead of the customary maximum of four, was isolated and dissected. Six of the spores survived, and exhibited the segregation shown in table 7. Three points should be noted in table 7: (1) Ascospores A629.2 and A629.3 did not give detectable conjugation figures, but also did not sporulate; (2) there are no spore pairs among the six spores as would be expected from a *Neurospora crassa* type of extra mitotic division; and (3) ascospores A631.1 to A631.4, included as a

sample of a four-spored ascus from this tetraploid, possess phenotypes consistent with those of the parental components, viz., $1a:3a$ from a cross of $a/a \times a/a$, 4+ : 0– for *Ur* from $-/- \times +/+$, and 2+ : 2– for *Tr*, *Me*, and *Ad* from $-/+ \times -/-$.

X-ray survival curves were run on ascospores A629.2, A629.3 and A629.4 (figure 2). Culture A629.3 gives a sigmoidal survival curve, extrapolating to about 2.2 at zero dose, which suggests that it is probably a diploid. Both

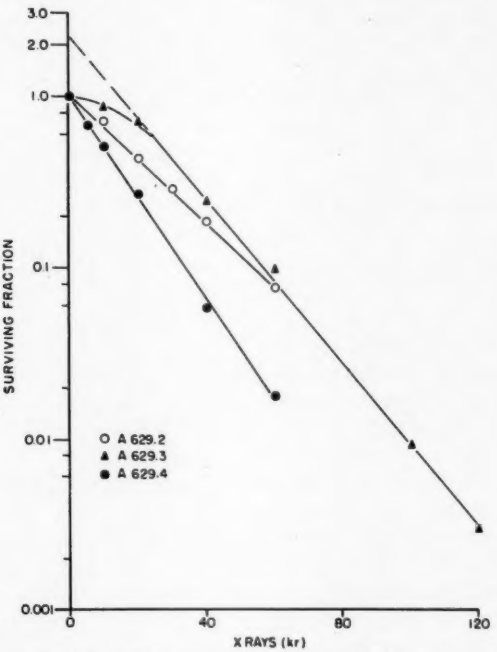

FIGURE 2.—Survival after X-irradiation of ascospores from seven-spored tetraploid ascus (A629).

A629.2 and A629.4 were killed exponentially. The slopes of the exponential curves place these two cultures in the class of "resistant haploids" or "exponential diploids" (POMPER and ATWOOD, in press). It is not possible to conclude with certainty, without genetic confirmation, that a culture giving an exponential survival curve is a haploid, since we have encountered diploids which give exponential curves. Therefore, we cannot ascribe a ploidy value to A629.2 and A629.4 without further genetic data. It should be noted that a six-spored ascus was encountered in a triploid culture, but only two spores germinated.

DISCUSSION

Matings between normally nonmating cultures to produce polyploids may come about in two ways: (1) through mutation of mating type, so that the cell is now compatible with the other member of the cross, and (2) through a mating between unchanged types. The demonstration of mutation of mating type in haploids (POMPER and MCKEE 1953) is the basis for postulating that this may have occurred in the cross of $a/a \times a/a$ which yielded a $4a:4a$ ratio. The mating between unchanged types, e.g., $a \times a/a$, might occur between a particularly potent a haploid and a diploid whose a gene is weaker than normal, so that the haploid is able to overcome the influence of the a component of the diploid. The solution to the problem of why an a/a diploid is normally nonmating remains obscure, but very challenging.

The observed yield of prototrophic colonies on a plate is small, presumably for the following reasons: (1) low frequency of conjugation, (2) delay in time before the rare conjugation occurs, so that environmental conditions are relatively unfavorable for further development, and (3) possible inhibition of the prototrophic cell by the auxotrophic cells. Therefore, both as a means of further studying the mating process and as an improvement in technique, it is suggested that a longer incubation period be employed before plating, and that a passage through liquid minimal be interspersed between the initial conjugation broth and the minimal plate.

The observation that presumably triploid clones after meiosis give rise to viable spores in good yield is indeed surprising. One would expect the meiotic progeny to be the victims of chromosomal imbalance, with consequent poor viability, yet five of the thirteen triploid clones analyzed gave at least fairly good germination. The behavior of the remaining eight is perhaps that to be expected for triploids, but makes analysis extremely difficult.

The fact that a given ascospore germinates and continues to grow suggests that its chromosome complement must be essentially normal. More direct evidence is given by the data on ascus A555. Three of its four spores have been shown to exhibit no genetic peculiarities. One would certainly expect that if the chromosome set had been imperfect, some evidence of it would have been forthcoming after sporulation of A555.3 and A555.4, or after crossing and sporulation of A555.2. Two alternative possibilities suggest themselves: (1) the number of chromosomes is so small that the probability of aneuploidy is low, or (2) a mechanism exists whereby at reduction division a complete diploid set goes to one pole, and a haploid set to the other, such that the second division then gives rise to two diploid and two haploid nuclei. Alternative (1) does not seem very likely in view of LINDEGREN's (1949) report of four to five linkage groups in *S. cerevisiae*, and our own failure to find linkage among five genes studied (unpublished). Alternative (2) requires a nonrandom distribution of the homologous chromosomes at reduction. The mechanism insuring this distribution may be lacking in the eight triploids which give poor ascospore germination.

A somewhat different way to look at the problem would be to think of the prototrophic isolates as heterocaryons, containing diploid and haploid nuclei. If this were so, one might visualize the diploid nuclei undergoing meiosis to produce four haploid nuclei, two of which would then fuse with the pair of haploid nuclei produced by a conjugate mitotic division of the original haploid component. For various reasons, we at present reject the heterocaryon hypothesis. First, plating survivors of X irradiation on minimal or minimal plus tryptophan, methionine, adenine, and uracil, reveals no difference in killing (POMPER and ATWOOD in press). If the cultures were growing vegetatively as heterocaryons, a significant difference in survival would be expected. Second, a cross such as the $a \times a/\alpha$ cross in table 1 should not yield any haploids independent for the four biochemical marker genes, but A555.1 is a completely prototrophic recombinant.

The aberrant ratios encountered in the triploid analyses cannot be explained with our present knowledge of the meiotic processes of these isolates. It does not appear to be simply a case of unstable genes, since (1) the same genes behave normally in segregations from diploids, (2) *Ad* and *Ur* were aberrant in two crosses (table 1), while *Tr* and *Ad* were aberrant in another (text), and (3) all of the marker genes segregated as expected in the cross analyzed in table 5. It should be noted that ascus A541 (table 1) can be analyzed similarly to A555 by using the genotype inferred for A541.4 from its behavior in a tetraploid cross, and from the observation that A541.1 is heterozygous for *Tr* and *Ur*, homozygous dominant for *Ad*, and homozygous recessive for *Me*. The ratios thus obtained are: Mating type ($4a:2\alpha$), *Tr* (2+:4–), *Me* (2+:4–), *Ad* (4+:2–), and *Ur* (4+:2–). These are identical with the ratios in table 4 for ascus A555, with the exception of the *Ur* segregation which was 3+:3–.

The recovery of four sporulating diploid ascospores from a cross of a known diploid by an inferred diploid (A541.4 × A555.3, table 6) is strong evidence in support of the belief that the prototrophic isolate was a tetraploid. A lack of difference between survival after X-irradiation on minimal and supplemented minimal agars renders unlikely the possibility that the prototrophic isolate propagates as a heterocaryon.

While uncontrolled sporulation of the diploid segregants from tetraploids (LINDEGREN and LINDEGREN 1951) was not observed; peculiarities of meiotic behavior were encountered. For example, the cross of two a/α diploids giving rise exclusively to ascospores possessing mating abilities, could hardly be predicted from a normal meiosis. Even more striking is the finding of an ascus with more than four surviving spores. It is possible that this observation is a clue to the problem of aberrant ratios in polyploids. It would be desirable to know whether the six surviving spores were indeed a mixture of haploids and diploids as suggested by the radiation inactivation curves. Asci of *S. cerevisiae* with more than four spores have been encountered in diploid material (WINGE and ROBERTS 1950; LINDEGREN and LINDEGREN 1953). As pointed out in the text, an extra mitotic division seems unlikely. With the

available data, it would be reckless to speculate on the mechanism whereby the supernumerary spores arose.

One should not overlook the possibility that some of the as yet unexplained segregation data from diploid yeast may have arisen through mechanisms similar to those giving rise to the anomalous polyploid segregation data. It would be logical to expect an increased frequency of such aberrancies in " unnatural " polyploid material as compared with " natural " diploid cells.

SUMMARY

Stable triploid and tetraploid clones of *Saccharomyces cerevisiae* have been isolated by a prototroph recovery procedure. Diploids heterozygous for mating type can apparently with rarity mate with haploids or other diploids to yield polyploids.

A triploid has been shown to segregate at meiosis into two diploid and two haploid spores. Support for this has been furnished by direct analysis through sporulation and crosses, and by X-ray survival data. A tetraploid has been shown to yield four sporulating diploid spores.

Segregation mechanisms, aberrant ratios, and the occurrence of asci with exceptional numbers of spores have been discussed.

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SOME ASPECTS OF THE CHEMICAL PROTECTION AGAINST RADIATION DAMAGE TO VICIA FABA CHROMOSOMES [1]

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SINCE 1933 when CRABTREE and CRAMER first reported that anaerobiosis decreased the effects of radium treatment on cancerous tumors, there have been numerous reports on the recovery of living cells, both animal and plant, from the effects of ionizing radiations. LEA (1945) and GRAY (1952) have reviewed the literature concerning these effects.

An excellent area for the study of these effects has been the production of chromosomal aberrations in plant tissues. THODAY and READ (1947) discovered that low oxygen tensions reduced the amount of chromosomal aberrations in actively growing *Vicia faba* roots; GILES and RILEY (1950) found a reduction in the chromosomal aberrations of Tradescantia microspores if the microspores were irradiated in nitrogen; and HAYDEN and SMITH (1949) showed a reduction of chromosomal aberrations in barley seed irradiated *in vacuo*.

The above work, however, is of theoretical interest only, as it seems impractical to keep aerobic organisms in oxygen-free atmospheres for lengthy periods of time. Thus much of the recent work has centered on the method of reducing the internal cellular oxygen tension by chemical means. Chief among the chemicals that do this are: sulfhydryl compounds, alcohols, glycols, and sodium hydrosulphite (BURNETT et al. 1951). In light of this, MICHAELSON (1952) was able to decrease the amount of chromosomal aberrations caused by X-rays in Tradescantia root tips by pretreatment with glutathione solutions. And RILEY (1952) was able to decrease the number of aberrations in onion roots by treating with sodium hydrosulphite.

Ostensibly, these chemicals work by reducing the number of active radicals formed in irradiated water as postulated by WEISS (1944, 1947) and thus they can protect against the "indirect effect" of ionizing radiations as first described by DALE (1943).

This present paper is concerned with the protective effects of some of the chemical reducing agents against chromosome damage in *Vicia faba*. The study is divided into three parts ascertaining 1) whether or not the agents can reduce damage if given to resting cells in the seed; 2) whether or not there is an additional effect of the chemicals beyond that of simple oxygen removal;

[1] These experiments were performed in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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3) whether or not it is the original breakage of the chromosomes or the restitution of ends already broken that the chemicals affect.

MATERIALS AND METHODS

Seeds of Argentine *Vicia faba* were used in all experiments. This particular organism is well suited for studies in radiation cytology for several reasons. Chief among these are 1) that there are but twelve chromosomes in a diploid cell, 2) the chromosomes are very large, 3) the root tips respond to the squash technique very well, and 4) by using seed, one is certain of irradiating all cells at resting stage and thus of avoiding the complications that arise due to the differential sensitivity of the various stages of mitosis to radiation.

The method of handling the seed was basically that of GRAY and SCHOLES (1951) with several major modifications. To decrease the amount of fungal infection, only unmarred seeds were used and these were first soaked in a 5% solution of calcium hypochlorite for thirty minutes. The seeds were then washed several times in distilled water to get rid of all traces of the oxidizer used as a disinfectant. The seeds were then soaked for twenty-three hours in glass-distilled water. The water was changed frequently during this period of time and to further decrease fungal infection the seed coat was removed as soon as it had softened.

The seeds were then transferred for exactly one hour, to solutions of the reducing agent to be tested. The solutions were all 2×10^{-3} M and made up with double-distilled water, the second distillation being done in an entirely glass system. This was necessary since some of the chemicals are rapidly oxidized by minute quantities of copper ions. The seeds were then transferred to sterile Petri dishes, the bottoms of which were lined with filter paper. The paper was moistened with the solutions and the seeds then immediately irradiated in this condition.

TheX-ray tube was operated at 160 KVP and 10 MA. All dosages were delivered at 200 r/min. as measured by a dosimeter in the radiation chamber at the actual time of irradiation. The only filtration utilized was that inherent in the tube itself and the Pyrex glass covers of the Petri dishes.

The seeds were then allowed to germinate on boards similar to those described by GRAY and SCHOLES. After a period of three days the roots were 2–3 centimeters long and the first mitotic divisions were occurring. One centimeter of each root was then cut off and placed for exactly seven hours into a 0.1% solution of colchicine to shorten the chromosomes and to collect metaphases. The root tips were then fixed in a solution of six parts ethyl alcohol, three parts glacial acetic acid, and one part chloroform. The latter was added to dissolve any oil in the cells that might obscure the chromosomes.

After at least three days in the fixative, aceto-carmine squash preparations were made of individual root tips. A detailed chromosome analysis was made of every scorable metaphase plate on the slide. Usually 3–8 slides were made of each treatment in order to get a large enough number of cells for statistical significance. The aberrations scored were rings and dicentric chromosomes

TABLE 1

Effect of chemical reducing agents on radiation-induced chromosome aberrations in Vicia faba. 500 r total dose at rate of 200 r/min. All chemical concentrations 2×10^{-3}M.

Treatment	No. cells	Normal	Rods & dots	Rings & dic. (2-hit)	% Rods & dots	% Rings & dic.
Water (no radiation)	200	199	1	....	....	....
Water	316	202	64	48	20.2 ± 2.6	15.2 ± 2.0
BAL	530	406	60	46	11.3 ± 1.4	8.7 ± 1.5
Cysteine	406	288	66	56	16.3 ± 1.8	13.8 ± 1.7
$Na_2S_2O_4$	354	256	64	37	18.0 ± 1.8	10.4 ± 1.6
Glutathione	236	162	48	38	20.3 ± 2.6	16.1 ± 2.5
Ascorbate	261	126	56	40	21.4 ± 1.5	15.3 ± 2.2

which are two-hit aberrations (SAX 1941). Since other workers have scored fragments in anaphase figures as an indication of chromosomal damage, the number of rod and dot deletions have been included to enable comparison of this experiment with others. However, as RICK (1940) has indicated, a proportion of the dot deletions are two-hit aberrations and so the number of rods and dots cannot be considered an accurate measure of one hit aberrations. The data were then computed on the basis of the percentage of cells containing the various types of aberrations. The " t " test of statistical significance was used both as a test of homogeneity for all slides receiving the same treatment and as a test of significance for different treatments. Fiducial limits were set up at the five percent level of significance.

RESULTS

In order to determine whether or not the chemical reducing agents were capable of decreasing the amount of radiation damage to seeds, the seeds of *Vicia faba* were soaked for one hour previous to irradiation in solutions of BAL, cysteine, sodium hydrosulphite, glutathione, or sodium ascorbate. They were then exposed to 500 r of X-rays delivered in two and one-half minutes. The results are presented in table 1.

TABLE 2

Effect of chemical reducing agents on radiation-induced chromosome aberrations of Vicia faba. In vacuo. 500 r total dose at rate of 200 r/min. All chemical concentrations 2×10^{-3}M.

Treatment	No. cells	Normal	Rods & dots	Rings & dic. (2-hit)	% Rods & dots	% Rings & dic.
Water (no radiation)	115	114	1	....	....	....
Water	215	169	31	17	14.4 ± 2.4	7.9 ± 1.8
BAL	392	314	58	25	14.6 ± 1.8	6.4 ± 1.2
Cysteine	268	208	41	20	15.3 ± 2.2	7.5 ± 1.6
$Na_2S_2O_4$	211	167	29	15	13.7 ± 2.4	7.1 ± 1.8
Glutathione	208	174−	28	11	13.4 ± 2.4	5.3 ± 1.6
Ascorbate	216	172	36	12	16.6 ± 2.5	5.6 ± 1.6

It will be seen that the BAL and the sodium hydrosulphite both afforded significant protection against the formation of two-hit chromosome aberrations It is interesting to note that these are the two chemicals that were found to be the most protective in bacteria (BURNETT et al. 1951).

Since these protective agents are reducing agents, they can decrease the internal oxygen tension of the cell and thus lower the amount of radiation damage. However, since some of these chemicals have other physiological properties, it was decided to test whether or not there was any additional effect of the chemicals after all the oxygen was removed. The seeds were, therefore irradiated *in vacuo*. The treatment was the same as in the previous experiment with the following exception. One-half hour before irradiation, the seeds were placed in the lucite tanks first described by KING, SCHNEIDERMAN and SAX (1952). They were then evacuated and the seeds were irradiated while in the vacuum. The results are presented in table 2.

TABLE 3

Effect of BAL (2×10^{-3}M) plus dosage fractionation on the production of radiation-induced chromosome aberrations in Vicia faba. 200 r/min. 3 doses with 1-hr. intervals.

Treatment	No. cells	Normal	Rods & dots	Rings & dic. (2-hit)	% Rods & dots	% Rings & dic.
Water (constant dosage)	549	278	173	109	31.6 ± 2.0	19.9 ± 1.7
Water (fractionated dosage)	500	253	164	92	32.8 ± 2.1	18.4 ± 1.7
BAL (constant dosage)	584	449	94	50	16.1 ± 1.5	8.6 ± 1.2
BAL (fractionated dosage)	658	381	102	36	15.5 ± 1.4	5.4 ± 0.9

None of the chemicals was able to decrease significantly the amount of chromosome aberrations obtained by irradiating in a vacuum. This indicates that the chemicals are only able to exert their protective effect when there is oxygen present in the cell. There is no additive effect noticed beyond that afforded by the direct removal of intracellular oxygen.

In view of the fact that some of the reducing agents are able to decrease the amount of aberrations induced by irradiation, the question arises as to the manner in which these chemicals work. Do they decrease the original breakage of the chromosomes or do they facilitate the restitution of ends already broken?

The classical method for the study of restitution is that which SAX (1941) proposed. That is to fractionate the radiation dosage, in which case the two-hit aberrations are decreased due to the restitution of broken ends.

Vicia faba seed seems to be an ideal organism on which to test the response of the chromosomes to fractionated dosages, since SAX and BRUMFIELD (1943) have hypothesized that the breaks remain open for a long period of time. The experiments were carried out as previously with the following changes. 600 r were given at the rate of 200 r/min. Those seeds that received

fractional dosage received three 200 r doses with one-hour intervals between treatments. The only chemical tested was the BAL which had proved to be the most effective in the earlier experiments.

The results are presented in table 3 and in figure 1.

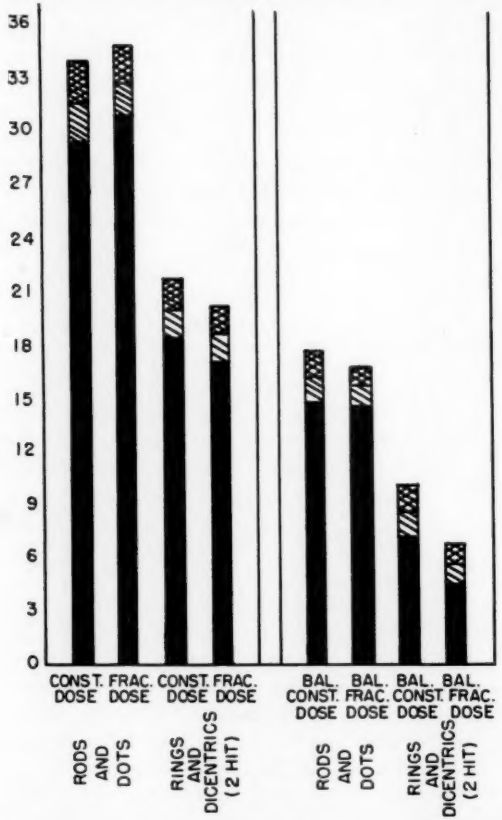

FIGURE 1.—Effect of BAL (2×10^{-3} M) plus dosage fractionation on the production of radiation induced chromosome aberrations in *Vicia faba*. All figures are $\pm$ 1 σ.

It will be seen that the fractionation of the dosage resulted in a significant decrease in the number of two-hit aberrations only in the presence of BAL.

DISCUSSION

As was pointed out previously both BAL and sodium hydrosulphite were able to protect against aberration production. However, it will be noted that the sodium ascorbate, another powerful reducing agent, gave no protection at all. This is in complete accord with studies made in other systems with this

particular chemical (PATT et al. 1950). It is therefore assumed that the ascorbate does not exert any action in those areas that are sensitive to the irradiation.

In light of the work done with bacterial (BURNETT et al. 1951) and mammalian systems (PATT et al. 1950), an anomalous result occurs when the seed is treated with cysteine. Significant protection is not attained. Since cysteine is in many respects a similar molecule to BAL one would certainly expect it to penetrate the plant cells if the BAL does. However, as was pointed out (BURNETT et al. 1951), cysteine has only one-half the protective value of BAL. This is what would be expected since the BAL has two SH groups whereas cysteine has only one. And indeed upon checking the rods and dots, one finds that although there is a statistically insignificant amount of protection, it is approximately one-half the amount of protection given by BAL. Even in the two-hit data if one varies the figure recorded by as little as one standard deviation, one gets one-half the protection of the BAL.

MICHAELSON (1952) in the only other attempt to test glutathione on plant chromosomes, found he could get significant protection only for fragments and not for anaphase bridges when he treated Tradescantia root tips. However he soaked the roots in glutathione for twenty-four hours, and it is quite probable that in the present experiments enough glutathione, a tripeptide, did not diffuse into the seed during the one-hour soaking.

It is quite evident none of the chemicals is able to give complete protection against the effects of the radiation. This is consistent with the results of all the other experiments ever performed on the protective effect of these chemicals.

It may be noticed that the placing of the seeds *in vacuo* caused no breakage to the chromosomes *per se*. Furthermore, as would be expected from the results of THODAY and READ (1947) and of HAYDEN and SMITH (1949), placing the seed in a vacuum resulted in protection from much of the damage.

According to the equations of WEISS (1944, 1947) the presence of the oxygen in the cells causes the formation of two compounds that would not be found in the absence of oxygen. These are the highly reactive hyperoxyl radical and hydrogen peroxide.

$$H + O_2 \rightarrow HO_2$$
$$2HO_2 \rightarrow H_2O_2 + O_2$$

It is quite logical, since these are the only two products that are excluded when water, which is omnipresent in cells, is irradiated anaerobically, that the chemicals protect by preventing the formation of these two substances. The other active radicals are present whenever the water is exposed to X-rays, anaerobically or not.

$$H^+ + OH^- \rightarrow H + OH$$

Since this is so and since the protective chemicals have no additive effect beyond oxygen removal as these experiments indicate, then it must be assumed that the chemicals are only able to protect against that portion of the "in-

direct effect" that is mediated through the hyperoxyl radical and hydrogen peroxide. The remaining chromosomal aberrations came therefore from two sources; 1) from that portion of the "indirect effect" mediated by H and OH radicals, and 2) from the direct ionization of the chromosome threads by the irradiation.

The question remains as to where the active radicals exert their influence. Do they break the chromosomes or do they inhibit restitution? Other groups of workers have already made contributions toward the solution of this problem (GILES and RILEY 1950; SCHWARTZ 1952; RILEY, GILES and BEATTY 1952; BAKER and VON HALLE 1953). However, they disagree as to the mechanism of lowering the oxygen tension. GILES and RILEY interpret their experiments on Tradescantia as indicating that oxygen affected the original breakage of the chromosomes. However, the possibility that the oxygen effect was due to a reaction on broken ends at the time of radiation was not excluded.

In contrast, both SCHWARTZ' work with endosperm mosaics in maize and BAKER and VON HALLE's work with recessive lethals in Drosophila indicate that it is the restitution process that is being affected.

The results of the present dosage fractionation experiments on *Vicia faba* indicate that the number of two-hit aberrations is dependent on total dosage and not on the method of administration. The two-hit aberrations are not decreased by fractionating the dosage as is the case in the classical Tradescantia experiments. This is consistent with the hypothesis of SAX and BRUMFIELD (1943) and is probably due to the fact that the ends remain open for periods longer than three hours.

Upon the addition of the BAL, however, fractionation results in a significant decrease of the two-hit aberrations. This is explainable if the addition of the chemical sped up the restitution time of the broken ends so that it was now less than the three hours in which the dosage was administered. This would then allow some of the breaks to become healed before other breaks occurred, and consequently would mean that there would be fewer breaks in the system at any given time that could form interchanges.

It therefore appears that the chemicals that reduce the oxygen tension within the cells increase the restitution rate of broken chromosomes. If one were to generalize from this hypothesis, then it would seem that the initial breakage hypothesis of GILES and RILEY is improbable, and that in the presence of oxygen the chromosomes probably react at the time of irradiation in some manner so as to decrease their ability to restitute.

SUMMARY

1) The seeds of *Vicia faba* were treated with 2×10^{-3} M solutions of BAL, sodium hydrosulphite, cysteine, glutathione, or sodium ascorbate, and then were given 500 r X-rays. The BAL gave good protection against radiation-induced chromosomal aberrations. The sodium hydrosulphite gave some protection but not as much as the BAL. The other chemicals did not enhance radioresistance.

2) The same chemicals were given and the seeds irradiated *in vacuo*. The vacuum gave partial protection against the production of aberrations. The chemicals had no additive effect over that of the vacuum.

3) The seeds were given 600 r of X-rays both in a constant dose and an intermittent one. There was no change in the number of aberrations when the dosage was fractionated. The experiment was repeated with the additional treatment of BAL. The BAL-treated seeds showed a decrease in the number of two-hit aberrations when the dosage was fractionated. This is interpreted as indicating that a reduction of the oxygen tension within the cells at the time of radiation increases the subsequent restitution rate of broken chromosome ends.

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THE MEIOTIC LOSS OF UNPAIRED CHROMOSOMES IN DROSOPHILA MELANOGASTER[1]

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IN cytological studies of species hybrids, ROSENBERG (1904) pointed out that univalents tend to be lost during meiosis. Since that time this has become a matter of common observation in plant studies, not only in species hybrids, which may represent a special case, but also in more normal situations in which univalents have been observed. In Drosophila, the suggestion that a chromosome which does not pair with its homolog might be lost during the meiotic divisions has been made by GERSHENSON (1933) to explain the discrepancy between the recovery of exceptional males and females resulting from primary non-disjunction in the male. Further evidence is presented below which supports the assumption that such loss does occur in Drosophila. In addition, information has been collected which bears on possible mechanisms of regular chromosome disjunction.

EVIDENCE FOR CHROMOSOME LOSS IN THE MALE

GERSHENSON (1933) found a large excess of exceptional males over exceptional females resulting from primary non-disjunction in the male in crosses of *bb* females to males carrying a *bb* deficient X chromosome and a normal Y chromosome. Since the recessive *bb* allele has a deleterious viability effect, this type of experiment has been repeated, using chromosomes now available which eliminate the viability complications of the earlier work.

Homozygous *y* females were crossed to four different types of males. In each of the types the X chromosome differed in the amount and distribution of heterochromatin. All four types carried the $sc^8 \cdot Y$ (MULLER 1948), a Y chromosome with a small duplication for the tip of the X from the $In(1)sc^8$ and carrying the normal allele of *y*. The X chromosomes used were: (1) a normal X marked with *y*; (2) the $In(1)sc^4$, a long inversion of the X which leaves most of the basal heterochromatin including *bb* and *Block A* proximally located ($= y\, sc^4$); (3) the $In(1)sc^8$, a long inversion of the X which places a considerable portion of the basal heterochromatin including *bb* and *Block A* distally ($= y^{31d}\, sc^8\, f\, v\, cv$); and (4) a chromosome (equivalent to the *bb* deficient chromosome of GERSHENSON) composed of the distal region of the $In(1)sc^4$ and the proximal region of the $In(1)sc^8$, and which is therefore deficient for a considerable portion of the basal X heterochromatin including the *bb* locus and *Block A* ($= y\, sc^4\, f\, v\, cv\, sc^8$). This chromosome is lethal in the

[1] This investigation was supported by a research grant C-1578C from the National Cancer Institute of the National Institutes of Health, Public Health Service.

homozygous or hemizygous state if no Y chromosome is present. The results from this series of experiments are given in table 1.

It is obvious from the data involving the sc^8 and $sc^4\ sc^8$ chromosomes that there is a large excess of the exceptional male class (y), which gets neither the X nor the Y chromosome from the father, as compared with the exceptional female class (+) which gets both the X and the Y. In addition, in those cases in which the X chromosome possesses the normal amount of heterochromatin (although possibly displaced) the frequency of primary non-disjunction in the male is very low, whereas in the case of the $sc^4\ sc^8$ chromosome, in which much of the basal X heterochromatin which is homologous with the Y chromosome is lacking, the frequency of primary non-disjunction is extremely high. These two observations suggest (a) that the high frequency of primary non-disjunction in the case involving the $sc^4\ sc^8$ chromosome is due to failure of pairing and consequent random segregation of the X and Y chromosomes at anaphase, and (b) that the deficiency in the recovery of both the X and Y chromosomes simultaneously (+ females) as compared with

TABLE 1

The results from crossing females homozygous for yellow to males whose X chromosomes differ in the amount and distribution of heterochromatin and which carry the sc^8-Y *(see text).*

Constitution of male parent	Phenotype of progeny			
	y (y^{31d})♀	+♂	+♀	y ♂
y	1,061	1,012	1	2
y sc^4	5,497	4,944	0	2
y^{31d} sc^8 f v cv	4,592	4,268	17	34
y sc^4 f v cv sc^8	2,839	1,649	160	1,376

neither (y males) is a function of the loss of one or both of these chromosomes during meiosis.

In addition to the discrepancy between the exceptional male and female classes in the cross involving the $sc^4\ sc^8$ chromosome, there is a large excess of the regular female class (y) as compared with the regular male class (+). In order to verify this observation, $sc^4\ sc^8$ males identical with those described above were mated to homozygous y attached X females which carried a normal unmarked Y chromosome. The progeny from such a mating would be: (1) $y\ cv\ v\ f$ males which get the X chromosome from the father and are in this respect equivalent to the y female class in table 1; (2) + females which get the $sc^8 \cdot Y$ from the father corresponding to the + male class in table 1; (3) $cv\ v\ f$ males which get both the X and the $sc^8 \cdot Y$ chromosomes from the father as does the + female class in table 1 (but which are XYY males and might have a reduced viability); and (4) y females which get neither the X nor the Y chromosome from the father and are therefore equivalent to the y male class in table 1. The progeny from this mating included 1,068 $y\ cv\ v\ f$ males, 464 + females, 56 $cv\ v\ f$ males, and 471 y females. A comparison of these results with those of the initial experiment is given in table 2. From these data

it would seem, therefore, that the discrepancy between the regular classes observed in the original experiment was real, since it is confirmed in this experiment, in which the distribution of mutants is different, and the sex of each class is reversed.

A possible explanation for this discrepancy is that the $sc^8 \cdot Y$ (perhaps when unpaired) acts in such a way as to lose the normal allele of *y*. This would change phenotypically + males to *y* males. Such loss might occur, for instance, by crossing over between the two arms of the $sc^8 \cdot Y$ chromosome, which would produce, as one of the complementary products, a Y chromosome or Y chromosome fragment lacking the normal allele of *y*, or it might happen by misdivision of the $sc^8 \cdot Y$ which would produce two metacentric Y chromosome fragments, one of which would be a duplication for the short arm of the Y and would lack the normal allele of *y*. In any case, barring complicated events in the $sc^8 \cdot Y$, inclusion of Y chromosomes or Y chromosome fragments not marked by the normal allele of *y* could be detected by the use of the normal allele of *bb* which is located on the short arm of the Y. Therefore, $sc^4\ sc^8$

TABLE 2

A comparison of the frequencies of sperm types produced by $y\,sc^4\,f\,v\,cv\,sc^8/sc^8 \cdot Y$ *males calculated from the mating to homozygous y females carrying two free X chromosomes (A), and the mating to homozygous y females carrying an attached-X chromosome and a normal Y (B).*

Type of X chromosomes from mother	Contribution of sperm			
	X	Y	X+Y	O
(A) Free	2,839 (47%)	1,649 (27%)	160 (3%)	1,376 (23%)
(B) Attached	1,068 (52%)	464 (22%)	56 (3%)	471 (23%)

males identical with those used in the experiments described above were mated to attached X females which did not carry a Y chromosome and which were marked with $y^2\ w^a\ bb$. The expected progeny from such a cross would be $y^2\ w^a\ bb$ females, of which there were 68; w^a females, of which there were 137; and *cv v f* males, of which there were 15. If Y chromosomes or Y chromosome fragments unmarked by the normal allele of *y* were being produced and included in the sperm nuclei, then there should result $y^2\ w^a$ non-*bb* females and *y cv v f* males. Neither of these two classes appeared in this experiment. Hence it would seem that the discrepancy between the regular classes in the initial experiment cannot be accounted for by the loss of the normal allele of *y* from the $sc^8 \cdot Y$. It will be shown below that this discrepancy can be accounted for by assuming chromosome loss.

A series of experiments was performed for the purpose of testing for the loss of unpaired chromosomes in a situation which did not involve primary non-disjunction. Homozygous *y* females were mated to males identical with those described above except that these males carried in addition to the $sc^8 \cdot Y$, a normal unmarked Y chromosome. The results from these experiments are given in table 3.

In an XYY male, the possibility of trivalent formation complicates the analysis, but irrespective of the types of disjunction, without chromosome loss one-half of the sperm should carry an X chromosome and the other half should not, and therefore the two sexes should be represented equally frequently in the next generation. This expectation is realized in each of the cases except the one involving the $sc^4\ sc^8$ chromosome. Here there is a decided deficiency of the female class (although the numbers are small due to the infertility of the parental males). Since in this case the homology between the X and Y chromosomes is very much reduced, pairing may often be of the YY type leaving the X chromosome unpaired. Such failure of pairing, if it resulted in the loss of the X chromosome, would produce a discrepancy in the recovery of the two sexes in the direction observed.

A similar argument may be applied to the recovery of the $sc^8 \cdot Y$, since once again, irrespective of the types of disjunction, one-half of the sperm should carry a $sc^8 \cdot Y$ (and produce phenotypically wild type offspring) and one-half of the sperm should not carry a $sc^8 \cdot Y$ (and produce phenotypically y offspring). However from the data involving the $sc^4\ sc^8$ chromosome it can be seen that there is a considerable deficiency of the non-y class.

TABLE 3

The results from crossing females homozygous for yellow to males whose X chromosomes differ in the amount and distribution of heterochromatin and which carry a normal Y chromosome in addition to the sc^8-Y.

Constitution of male	Phenotype of progeny			
	y (y^{31d})♀	+♂	+♀	y♂
y	1,464	1,429	851	903
$y\ sc^4$	1,993	1,962	1,381	1,437
$y^{31d}\ sc^8\ f\ v\ cv$	801	861	770	870
$y\ sc^4\ f\ v\ cv\ sc^8$	66	133	44	229

ESTIMATES OF THE RELATIVE FREQUENCIES OF LOSS

It has been shown above that as far as can be genetically determined the discrepancy between the regular classes in the crosses involving $sc^4\ sc^8/sc^8 \cdot Y$ males was not the result of loss of the normal allele of y from the $sc^8 \cdot Y$. If, then, the regular male class and the regular female class be taken as truly indicative of the recovery of the $sc^8 \cdot Y$ chromosome and $sc^4\ sc^8$ chromosome respectively, the Y chromosome is being recovered only 58 percent (1,649/2,839) as often as the X. Since, in this case, the X and Y chromosomes are presumably unpaired with exactly the same frequency, the difference in their recovery would seem to represent a differential ability of the unpaired X chromosome and unpaired Y chromosome to become included in the products of the first meiotic division. This 42 percent loss of the Y chromosome relative to the X chromosome is for all cells; it must therefore represent a minimum estimate of the loss occurring in that fraction of the cells having unpaired chromosomes. This is so for the reason that the total must include a sizable

fraction of cells in which disjunction is normal which would tend to obscure the extent of the loss in cells with unpaired chromosomes. An estimate of the actual frequencies of loss can be arrived at in the following way. In the male, disjunction may either be regular or irregular, and these two types will occur with unknown frequencies of R and $(1-R)$ respectively. Assuming that there is no chromosome loss when disjunction is regular, one half of the gametes from such disjunction will carry an X chromosome and the other half will carry a Y chromosome. In the case of irregular disjunction, if it is assumed that the X and Y chromosomes move at random with respect to one another such that the two chromosomes separate as often as they proceed to the same pole, then, without chromosome loss, there should be produced X, Y, XY, and nullo-XY sperm, each with a frequency of $\frac{1}{4}(1-R)$. However, chromosome loss can change potential gametes of one type into recoverable gametes of another type. For instance, a potential XY-bearing sperm would be recovered as an X-bearing sperm if the Y chromosome were lost and the X chromosome were not lost. Chromosome loss can be taken into account if it is assumed that the frequency of loss for either chromosome is the same irrespective of whether the two chromosomes proceed to the same or to opposite poles in the cells in which disjunction is irregular. Let L_X and L_Y equal the frequencies of X chromosome and Y chromosome loss respectively. If this is done, then the *y* female class arises from the fertilization of X-bearing eggs by X-bearing sperm which come from that half of the products of regular disjunction which receive an X chromosome, $\frac{1}{2}R$; from that fraction of the potential X-bearing sperm from irregular disjunction in which the X chromosome is not lost, $\frac{1}{4}(1-R)(1-L_X)$; and finally from that fraction of the potential XY-bearing sperm from irregular disjunction in which the Y chromosome is lost and the X chromosome is not lost, $\frac{1}{4}(1-R)L_Y(1-L_X)$.

Summing,

$$y\ ♀♀\ (=.4712) = \tfrac{1}{2}R + \tfrac{1}{4}(1-R)(1-L_X) + \tfrac{1}{4}(1-R)L_Y(1-L_X).$$

Similarly,

$$+\ ♂♂\ (=.2737) = \tfrac{1}{2}R + \tfrac{1}{4}(1-R)(1-L_Y) + \tfrac{1}{4}(1-R)L_X(1-L_Y)$$
$$+\ ♀♀\ (=.0265) = \tfrac{1}{4}(1-R)(1-L_X-L_Y+L_X\cdot L_Y)$$
$$y\ ♂♂\ (=.2284) = \tfrac{1}{4}(1-R) + \tfrac{1}{4}(1-R)L_X\cdot L_Y + \tfrac{1}{4}(1-R)(L_X+L_Y).$$

The solution to these equations gives $R = 49.3\%$; $L_X = 0.9\%$; and $L_Y = 78.9\%$. The low calculated frequency of loss of the X chromosome in this case seems strange because this same X chromosome in an XYY male appears to be lost with an approximate frequency of 53 percent, which may be arrived at simply by obtaining the percentage discrepancy between the observed recovery of females and the expected 50 percent. Of the total of 472 progeny (see table 3) 236 should have been female, but only 110 were; presumably 126 X chromosomes were lost and 126/236 equals 53 percent. A figure arrived at in this way represents the total loss of the X, and hence (as above) is a minimum

figure for loss of the unpaired chromosome, assuming that loss occurs predominantly when the chromosome has not paired.

This may indicate that the $sc^4\ sc^8$ X chromosome is actually lost with a different frequency in XY as compared with XYY males, since certain comparisons can be made which are independent of the assumptions upon which the above analysis is based, but which give results that agree closely with the figures arrived at in that analysis. The overall loss of the X chromosome can be arrived at directly from the sex ratio, since, assuming no loss of the X chromosome, females should be recovered as one-half of the progeny. In the experiment involving $sc^4\ sc^8/sc^8 \cdot Y$ males, the females constituted 49.8 percent of the progeny indicating that loss of the X chromosome in this case must indeed be very low. In addition, the total loss of the $sc^8 \cdot Y$ chromosome can be estimated, since, assuming no loss, one-half of the progeny should be y and the other half non-y. In this experiment the non-y class represented only 30 percent of all the progeny which represents a total loss of the $sc^8 \cdot Y$ of 40 percent ($100\% - 30\%/50\% = 40\%$). Since, however, this figure includes those cases in which the $sc^8 \cdot Y$ was recovered from normal disjunction as well as the cases in which the $sc^8 \cdot Y$ was unpaired, a correction must be made to get the frequency of loss of the unpaired Y (L_Y). If R equals 49 percent, then L_Y is approximately equal to twice the total frequency of loss, or 80 percent.

The frequency of loss of the unpaired $sc^8 \cdot Y$ in the XYY male cannot be calculated because it is impossible to estimate the frequency with which this chromosome is unpaired. However, the total loss can be estimated and, as in the previous cases, such an estimate must represent the minimum loss. In this case the total loss of the $sc^8 \cdot Y$ is equal to 24 percent ($100\% - 38\%/50\% = 24\%$). It should be noted that this represents loss of only one of the two Y chromosomes in this male. The other Y chromosome is presumably lost with a similar frequency, but such loss would be undetectable in this experiment.

EVIDENCE FOR CHROMOSOME LOSS IN THE FEMALE

If in an XXY female the Y chromosome is lost with some frequency, there should result a deficiency of Y-bearing eggs as compared with the expected 50 percent. To test for such loss, females homozygous for y but carrying the $sc^8 \cdot Y$ (and therefore phenotypically wild type) were crossed to $y\,B$ males which carried the attached XY chromosome (LINDSLEY and NOVITSKI 1950). This chromosome is an entire X with the long arm of the Y chromosome (YL) attached to the centromere, and the short arm (YS) attached to the tip. The chromosome is therefore symbolized YSX · YL, and contains all the essential elements of the X chromosome plus all the fertility factors of the Y chromosome.

Males of this same constitution were crossed to females identical with those just described, but in which the $sc^8 \cdot Y$ had been replaced by FR2 (NOVITSKI 1951), a fragment equivalent to the long arm of the Y and carrying the normal allele of y. The results from these two experiments are given in table 4.

The situation in an XXY female is complicated by the possibility of trivalent

formation which makes it impossible to calculate the frequency with which the Y chromosome is unpaired. However, certain comparisons can be made between the complementary regular classes which result from those cases in which the two X chromosomes separate, since these classes must come almost exclusively from instances in which the two X chromosomes were paired. This is so for the reason that one-half of all the progeny from cases in which the X and Y chromosomes were paired would be exceptional males, and in this experiment there were only approximately 5 percent of secondary exceptional males. In addition, if in those cases in which the X and Y chromosomes were paired there were loss of the unpaired X, then the exceptional male class would be further increased. Therefore, since the regular classes appear to result predominantly from cases in which the two X chromosomes were paired, if the $sc^8 \cdot Y$ chromosome is not lost, one half of the regular females and one half of the regular males should be *y*, the other half non-*y*. Loss of the $sc^8 \cdot Y$ would be reflected as a deficiency of the non-*y* class.

In the cross involving the females which carry the $sc^8 \cdot Y$, it can be seen that the non-*y* females are recovered 60 percent as frequently as the *y* females

TABLE 4

Results from crossing y/y/sc^8·Y and y/y/FR-2 females to YSX·YL, y B males.

Constitution of female	Phenotype of regular progeny			
	y B ♀	*B* ♀	*y* ♂	+ ♂
y/y/sc^8·Y	1,214	725	1,360	1,021
y/y/FR-2	1,279	932	1,674	1,265

(725/1,214), while the + males are recovered 75 percent as often as the *y* males (1,021/1,360).

The discrepancy between the *y* and non-*y* female classes can possibly be explained in part by the fact that the non-*y* females carry the Y chromosome arms of the YSX·YL from the male parent and the free $sc^8 \cdot Y$ from the female parent, and therefore contain the heterochromatic equivalent of two Y chromosomes. Such an excess of heterochromatin might have an effect on the viability of the female as has been shown previously (NOVITSKI 1954). The excess of *y* males as compared with + males, however, would seem to be the result of some factor other than viability since the equality of the regular classes resulting from crosses of homozygous *y* females to $y/sc^8 \cdot Y$ males (see table 1) indicates that the $sc^8 \cdot Y$ has no deleterious effects in an $X/sc^8 \cdot Y$ male. In addition, if the $sc^8 \cdot Y$ is replaced by FR2 which is cytologically a rod, and which lacks some, if not all, of the fertility factors of the short arm of the Y and therefore, presumably consists of the long arm of the Y only, the difference in the comparison between male classes as compared with female classes disappears. This indicates that the difference in these comparisons observed in the previous experiment was due to a viability effect of excess heterochromatin. In this experiment the non-*y* females are recovered 73 per-

cent as often as the *y* females (932/1,279), while the + males are recovered 76 percent as frequently as the *y* males (1,265/1,674). The depression of the non-*y* classes, which cannot be accounted for by a viability effect, might be interpreted as a loss of the $sc^8 \cdot Y$ (or FR2).

TESTS FOR THE LOSS OF COMPOUND CHROMOSOMES

The evidence presented above suggests that unpaired chromosomes can be lost, and occasionally with a fairly high frequency. In this connection there are two chromosome types that are of particular interest because their disjunctional behavior can be studied both with and without a homolog. The chromosomes are the attached X and the attached XY (= YSX · YL; for description see above). Tests for loss of these chromosomes were carried on in the female and male respectively.

For the purpose of testing for loss of the attached X chromosome, females carrying an attached X homozygous for *y* and carrying no Y chromosome were mated to Canton-S (wild-type) males. The progeny included 3,716 *y* females and 4,056 + males, an 8 percent excess of the male class. A control experiment in which the females carried, in addition to the attached X described above, a normal Y chromosome which should supply a pairing partner for it gave 3,434 *y* females and 3,820 + males, a 10 percent excess of the male class. From these data there appears to be no appreciable difference between the recovery of the attached X chromosome with and without a homolog.

To test for loss of the YSX · YL chromosome, males carrying this chromosome marked with *y B*, both with and without a homolog, were crossed to females homozygous for *y*. In the control experiment the males carried in addition to the YSX · YL, *y B* chromosome, FR2 (a fragment equivalent to YL and marked by the normal allele of *y*; see above). In this experiment non-disjunction in the male gives results that are distinguishable from the regular classes and from non-disjunction in the female. There were only 7 cases of non-disjunction in the male from a total number of 7,181 flies recovered in this experiment, indicating that FR2 regularly pairs with the YSX · YL chromosome. The regular classes consisted of 3,652 *y B* females and 3,518 + males, approximately a 3 percent excess of the female class. The experimental set in which the males carried the YSX · YL, *y B* chromosome without a homolog yielded 3,095 *y B* females and 3,498 *y* males, approximately an 11 percent depression of the female class. The total difference between the two sets then represents a 14 percent deficiency in the recovery of the YSX · YL chromosome when it has no pairing partner. A figure arrived at in this way may well be open to doubt. However, estimates for the frequency of loss of this chromosome can also be obtained from the crosses of X/X/Y and X/X/FR2 females to males carrying the YSX · YL, *y B* chromosome (see table 4). In these experiments eggs bearing a single X chromosome will produce *y* males when fertilization is by nullo-XY sperm, and *y B* females when fertilization is by XY-bearing sperm. Loss of the YSX · YL chromosome, therefore, should be reflected as a deficiency of *y B* females as compared with

y males. Reference to table 4 shows an 11 percent (100% – 1,214/1,360) deficiency in the recovery of the YSX · YL chromosome in the cross involving females carrying the $sc^8 \cdot Y$, and a 24 percent (100% – 1,279/1,674) deficiency in the cross in which the female carried FR2.

INTERPRETATION OF ATTACHED X RESULTS

The fact that the disjunctional behavior of the attached X chromosome appears to be the same irrespective of whether or not a homologous chromosome is present is of interest in that it brings evidence to bear on the question of whether chromosome loss is conditioned by unpaired centromere regions, unpaired chromatin, or both. This is so for the reason that the arms of the attached X pair normally with each other (as is evidenced by the fact that crossing over is normal in attached-X experiments), but the centromere region is of necessity always unpaired if no homologous chromosome is present. Since, then, the attached-X chromosome without a Y does not appear to be lost with any appreciable frequency, it would seem as though regular disjunction is not dependent upon the pairing of centromere regions, but is presumably dependent upon pairing somewhere along the length of the chromosome, most probably the basal heterochromatin, since centric heterochromatic fragments (e.g., FR2) do disjoin normally.

Another argument may be applied in support of the view that pairing at the centromere region is not necessary for regular disjunction. The level of primary non-disjunction is very low in males with a sc^8 X chromosome, but high in males with a $sc^4\ sc^8$ X chromosome, and the only difference between these two chromosomes is the presence of a more extensive heterochromatic section located distally on the sc^8 chromosome. Apparently the Y chromosome can pair with, and disjoin from, this distal segment. Under these conditions the centromere regions would be unpaired.

ANALYSIS OF LOSS OF THE YSX · YL CHROMOSOME WITH RESPECT TO THE BEHAVIOR OF THE TANDEM METACENTRIC COMPOUND X CHROMOSOME

The values for the frequency of loss of the YSX · YL chromosome arrived at above account for two inconsistencies in data from NOVITSKI and BRAVER (1954) on the tandem metacentric compound X chromosome, also known as the tandem attached X. In those experiments the compound X chromosome was heterozygous for an inversion, *dl-49*, which suppressed crossing over so that double exchanges were very rare and can be disregarded here. As shown in figure 1, there are two distinctly different kinds of single exchanges, one of which (A) produces a single ring chromosome and the other (B) a dicentric. These dicentrics produce lethal zygotes (see STURTEVANT and BEADLE 1936 and NOVITSKI 1951) and so are undetectable. However, an estimate may be made of their frequency since exchanges of type A and B should occur equally frequently.

Females carrying the tandem metacentric and a fragment which disjoins regularly from it were mated to males carrying the YSX · YL, *y B* chromo-

some. The various types of progeny produced from this mating are given in figure 1, and the results in table 5. There are two independent comparisons that can be made. In the first place the rings newly formed by crossing over should be recovered both as males and females and these two classes should occur equally frequently. However, 322 were recovered as females and 390 as males; this deviation from the expected 1 : 1 can hardly be explained by differential viability since the males carried more mutants than the females in most cases. If it is assumed that the females, which get the YSX · YL chromo-

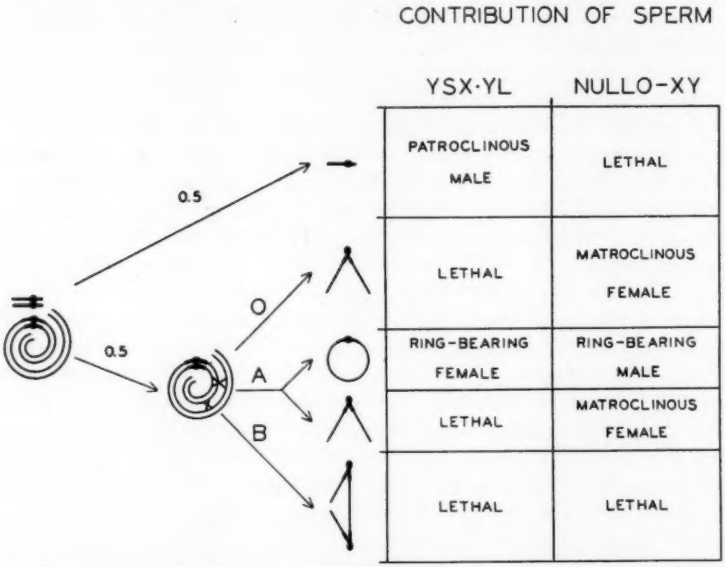

FIGURE 1.—The types of progeny expected from a cross of females carrying the tandem metacentric compound X chromosome and a fragment which disjoins regularly from it to males carrying the attached-XY (= YSX · YL) chromosome. The consequences of no exchange (0) and the two types of single exchanges (A and B) in the compound chromosome have been included; the results of double exchanges have been omitted.

some, are being depressed because of the loss of that chromosome in the parental male, then the frequency of loss is 17 percent.

A second comparison involves a calculation of the total number of zygotes in the experiment which resulted from fertilization by XY-bearing sperm as compared with those which resulted from fertilization by nullo-XY sperm. The estimate of the number of zygotes that resulted from fertilization by XY-bearing sperm comes from twice the number of recovered patroclinous males since, as can be seen in figure 1, one-half of all eggs should be nullo-X and are recoverable only when fertilized by an XY-bearing sperm. The total number of zygotes arising from fertilization by such sperm then becomes 5,608 ($2 \times 2{,}804$).

An estimate of the number of eggs fertilized by nullo-XY sperm is arrived at by calculating the exchange frequency in the following way. The 390 ring-bearing males carry rings which have been shown to disjoin non-randomly from the non-crossover tandem metacentric chromatid (NOVITSKI 1951). The best estimate for tetrads with an exchange of type A is $2\ (3/2 \times 390) = 1{,}170$. This figure then represents the frequency of dicentrics which come from an exchange of type B. Of the 2,456 matroclinous females, 585 – 390, or 195, come from exchange tetrads leaving 2,261 as the number coming from no exchange tetrads. Reference to figure 1 will show that the total number of zygotes resulting from fertilization by nullo-XY sperm must be $2 \times 2{,}261 + 2 \times 1{,}170$ (= 6,862). The difference between this and the figure arrived at for the total number of zygotes arising from fertilization by XY-bearing sperm (5,608) is 18 percent.

TABLE 5

Types of progeny produced from the mating of females carrying a fragment and the tandem metacentric compound X chromosome heterozygous for an inversion to YSX·YL, y B males. (Data from Novitski and Braver 1954.) Double crossovers are very rare and have been omitted from this table. The fragment carried in the parental female has the normal allele of y, and hence the patroclinous males are non-y.

Type of progeny	Phenotype of progeny	Number of progeny
Patroclinous ♂♂	*B*	2,804
Matroclinous ♀♀	*y*	2,456
Ring-bearing ♀♀	*y B*	322
Ring-bearing ♂♂	*y m f car*	390
	y v f car	
	y m car	
	y m	

The fact that the YSX · YL chromosome without a homolog is lost much less often than an unpaired Y chromosome might be accounted for by assuming that normally the long arm of the Y chromosome which is attached to the centromere of the YSX · YL chromosome bends back and pairs with the basal heterochromatin of the X, but that there is occasional failure of such pairing giving rise to the observed frequencies of loss.

DISCUSSION

Chromosome loss in *Drosophila melanogaster* had been suggested as early as 1925 to explain an average of four recovered exceptional males to one recovered exceptional female resulting from primary non-disjunction in the female (MORGAN, BRIDGES and STURTEVANT 1925). The suggestion at that time, however, was that "primary non-disjunction is initiated by some unusual local difference, in consequence of which the synapsed X's lag upon the reduction spindle and separate from each other only with difficulty." The present work is more in accord with the alternative possibility that primary

non-disjunction results from occasional failure of the X chromosomes to pair, with the consequent loss of one or both of these causing a discrepancy in the recovery of the exceptional classes in the direction observed.

From experiments involving XXY females and XYY males, BRIDGES (1916) suggested that: (1) in XXY females pairing is always between two of the three homologs (preferentially between the two X's) which disjoin normally, while the unpaired chromosome proceeds to one or the other of the poles and is always included in a daughter nucleus, and (2) in an XYY male pairing is again between two of the homologs (but at random) which disjoin normally while the unpaired chromosome again proceeds to one pole or the other and is always included in a sperm nucleus. However, if an unpaired X chromosome does tend to be lost during meiosis (and with a detectable frequency) then the equality of the sexes in the crosses involving XYY males (table 2), in all but the sc^4 sc^8 case, would indicate that the X chromosome is paired in almost all, if not all, meioses. This pairing either could be between only two of the homologs as BRIDGES postulated, or could involve all three chromosomes in a trivalent configuration (see COOPER 1948). It may be noted that if BRIDGES' interpretation were correct, then, in all but the sc^4 sc^8 case, pairing would have to be almost exclusively between the X and the Y with homosynapsis a rarity. This, however, should result in the loss of the unpaired Y chromosome. From the data given in table 3 there is no evidence for such loss.

With respect to the sc^4 sc^8 chromosome, GERSHENSON (1933), from crosses involving sc^4 sc^8 XYY males, concluded that pairing was predominantly of the YY type with consequent loss of the unpaired X; a conclusion with which the results of this study agree. However, in that same work it was reported that X and Y sperm were produced in equal numbers by sc^4 sc^8 XY males. In this respect the two sets of data do not appear to be comparable, perhaps because the frequencies of non-disjunction are different in the two experiments.

Finally it should be noted that although evidence for the loss of unpaired chromosomes has been found in both males and females, there is no evidence to indicate that the mechanism involved in these two cases is the same, especially since the meiotic behavior of males and females in Drosophila differs in many respects.

SUMMARY

Four different types of XY and XYY males were tested for loss of their X and Y chromosomes. The types differed in the amount and distribution of heterochromatin carried on the X chromosome. The evidence indicates that loss of these chromosomes occurs only in those cases in which the homology between them is very much reduced, indicating that chromosome loss occurs predominantly when the chromosomes are unpaired. The frequencies of loss for those chromosomes which showed loss are as follows: (1) the Y chromosome in an XY male, 42 percent; (2) the X chromosome in an XY male, 0.3 percent (not significantly different from 0); (3) the X chromosome in an XYY male, 53 percent; (4) one of the Y chromosomes in an XYY male, 24

percent. Tests were also run to determine the frequency of loss of a Y chromosome and a Y chromosome fragment in XXY (and Y fragment) females. The frequency of loss in both of these cases was 25 percent. These frequencies, which represent the total loss of the chromosome, must be minimum estimates of the loss of the unpaired chromosome.

The attached XY chromosome is lost with a frequency that varies from 11 percent to 24 percent. The application of this loss to previously published data on the tandem metacentric compound X chromosome is discussed in detail.

The data are in accord with the idea that primary non-disjunction in the female results from occasional failure of the X chromosomes to pair, with the consequent loss of one or both of these resulting in the observed discrepancy between the recovery of primary exceptional males as compared with primary exceptional females.

From the data taken as a whole, and particularly from the evidence indicating that the attached X chromosome is not lost in the absence of a homolog, it is suggested that normal disjunction is dependent upon the pairing of heterochromatic regions and is independent of pairing at the centromere region.

ACKNOWLEDGMENT

The authors wish to express their appreciation to DR. E. NOVITSKI for his help and criticisms throughout the course of this investigation.

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ARTIFICIAL AND NATURAL HYBRIDS IN THE GRAMINEAE, TRIBE HORDEAE. VII. HYBRIDS AND ALLOPOLYPLOIDS BETWEEN ELYMUS GLAUCUS AND SITANION SPP.

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IN the first paper of this series (STEBBINS, VALENCIA and VALENCIA 1946a) two different natural hybrids between *Elymus glaucus* and *Sitanion jubatum* were described. Both were intermediate between their parents and were completely sterile. They nevertheless had essentially normal meiosis, with the chromosomes forming 14 bivalents or occasionally 13 bivalents and 2 univalents at first metaphase. During the past seven years a series of artificial hybrids has been made between various strains of *E. glaucus* and *S. jubatum* as well as the closely related *S. hystrix*. Because of the relative vigor of *E. glaucus* and the extreme drought resistance of many strains of *Sitanion*, the senior author has hoped that progeny from such hybrids would yield vigorous, drought-resistant grasses for revegetating the dry range lands of California. For this purpose, most of the hybrids have been treated with colchicine in order to produce allopolyploids from them.

The results of these experiments have revealed a remarkable array of different types of behavior after hybridization between different strains supposedly belonging to the same taxonomic species. In some instances the F_1 hybrids were easily obtained, vigorous, and yielded allopolyploids which were highly fertile in the doubled sectors of F_1 plants and gave second generation progeny (C_2) containing many vigorous, fertile plants. At the other extreme are examples of hybridizations which failed even after repeated attempts and of F_1 plants which were so weak that they did not flower. The present contribution presents these results, along with preliminary considerations regarding their interpretation.

The cytological studies of the allopolyploids were made by the junior author during the year 1951–1952. The initial hybridizations and the morphological studies of the hybrids were made by the senior author and his assistants.

MATERIAL AND METHODS

The parental strains are described, with notes on their origin, in table 1. In this paper, each strain is designated by a place name, which is italicized in the table. Hybridizations were made according to the method described earlier (STEBBINS and TOBGY 1944). The F_1 hybrids were treated with colchicine as young plants according to the method described by STEBBINS (1949). Treatments of clonal divisions with 0.2% colchicine for four hours gave best

TABLE 1

Origin and description of the parental strains.

Species and culture numbers	Locality of origin	Morphological characteristics
Elymus glaucus		
3987,* 073, 166, 229, 328	Dry hills, in chaparral, west of *Lakeport*, Lake County	Low growth, narrow leaves, dense tuft, small spikelets.
881, 015, 115, 228	*Sebastopol*, Sonoma County (USSCS no. P10128	Tall, vigorous, medium broad leaves, spikelets medium.
3909, 038, 117, 230	Open pasture, *Orinda*, Contra Costa County	Coarse, heavy anthocyanin pigmentation, sheaths hairy, spikelets large.
3978, 139	Steep bluff facing ocean, *Carmel* Highlands, Monterey County	Loose tufts, stems with many nodes, lemmas awnless (near *E. virescens* Trin.).
639, 837, 943, 282	North facing hillside, under oaks, *Big Sur*, Monterey County	Vigorous growth, dense tufts, spikelets medium sized.
4086, 231, 317	Grazed pasture, Trescony Ranch, *San Lucas*, Monterey County	Moderate height and vigor, pale green leaves, spikelets medium sized.
2865, 404, 835, 937, 118	Steep north facing slope, north of *Santa Paula*, Ventura County	Moderate vigor, leaves thin and quickly dormant, spikelets medium sized.
3965, 014, 114	Shady bottomland along Cosumnes River west of *Wilton*, Sacramento County	Tall vigorous, broad leaved, spikelets medium sized.
3790, S100, 087, 119	Base of steep north facing bluff along Stanislaus River, west of *Knight's Ferry*, Stanislaus County	Similar to Wilton.
Sitanion jubatum		
3473, 769, 979	Sandy, open wash, northeast of *Redding*, Shasta County	Relatively tall, awns shorter than in most *S. jubatum.*
969, 121	Between road and railroad, dry soil, *Nimbus*, Sacramento County	Tufts loose, with few sterile shoots, leaves narrow, elongate, divisions of glumes very numerous.
402, 580, 622, 1133, 283	Along railroad right of way, dry soil, north of *Madera*, Madera County	Low growth, numerous heads, leaves very hairy, spikelets intermediate.
3897, 019, 120	Along roadside west of *Caliente*, Kern County, very dry soil	Very narrow leaves, medium height, awns rather long.
3895, 971, 018, 249	Dry roadside, near Monolith Cement Works, east of *Tehachapi*, Kern County	Similar to Caliente, but forms denser tufts with numerous sterile shoots.
3883, 970, 017	Steep north facing slope in desert canyon east of *Bishop*, Inyo County	Poor growth in coastal California, nearly glabrous, glumes with many divisions, but shorter and less stiff than in typical *S. jubatum.*
Sitanion hystrix		
3996, 113, 247	Dry, exposed south facing desert slope along Carson River west of *Fort Churchill*, Nevada	Very small plant, smooth leaves, short awns, heading profusely.
3877, 972	Dry soil among sagebrush, mouth of *Leevining* Canyon, Mono County	Taller than Ft. Churchill, leaves densely short hairy, spikelets resembling Ft. Churchill.

*Numbers in italics represent the original field collection numbers, according to which specimens are filed in herbaria.

results; in some instances very good results were obtained by repeating this treatment on two consecutive days. As a rule, the treated plants were not examined cytologically, but the sectors with doubled chromosome number were recognized by their fertility. The success of this treatment was very variable; in some instances as many as 80% of the treated divisions contained doubled sectors, while in other instances no doubling was detected at all, although as many as 30–40 divisions were treated and raised to maturity.

Cytological preparations were made by the aceto-carmine method from fixations in 3 parts absolute alcohol : 1 part glacial acetic acid. Drawings were made with a camera lucida, and are reproduced at a magnification of 1500×. Because of the high number of chromosomes and their tendency to clump, analysis of chromosome configurations at I metaphase was difficult, and only selected, well-spread cells could be studied. For this reason, it is possible that cells containing the most complex series of configurations were not analyzed, but since this difficulty was general, it does not affect comparisons between different allopolyploids in respect to chromosome behavior.

CROSSING BEHAVIOR OF THE PARENTAL STRAINS

The first unexpected result of the present work is the great difference between the various hybrid combinations attempted in the degree of success of the cross, as illustrated in figure 1. In the most successful crosses, more than twenty hybrid seeds were obtained out of sixty florets emasculated and pollinated; a result which approximates 50% of the seed setting obtained in cross pollinations between plants of the same strain. On the other hand, no hybrid seeds at all were obtained in 13 out of the 35 crosses attempted, and in 4 more the F_1 hybrids were so weak that they did not reach maturity. In several instances, the number of florets emasculated and pollinated was too small to provide positive evidence that the combination concerned is impossible. In some combinations, such as Lakeport × Fort Churchill, Orinda × Caliente, Carmel × Redding, Carmel × Fort Churchill, and Wilton × Nimbus, repeated attempts in both directions produced no hybrids, indicating at least that these crosses are very difficult to make.

A glance at figure 1 reveals the fact that the ease of crossing within this group bears no clear relationship to the place of origin or the systematic relationships of the parental strains. In both their habitat and their external morphology the two strains of *S. hystrix* from east of the Sierra Nevada are considerably more different from the coastal and foothill strains of *E. glaucus* than are the strains of *S. jubatum* which come from the valley and foothill area. Yet out of 10 crosses attempted with *S. hystrix*, 7 yielded vigorous F_1 hybrids, while out of the 25 crosses attempted with *S. jubatum*, only 12 yielded vigorous F_1 hybrids. The ability of some sympatric strains of *E. glaucus* and *S. jubatum* to intercross rather easily is evident from the frequent occurrence of natural hybrids. On the other hand, there appear to be differences between strains of both *E. glaucus* and *Sitanion* spp. in their general ability to form hybrids with the opposite "genus." For instance, the Sebastopol strain of *E.*

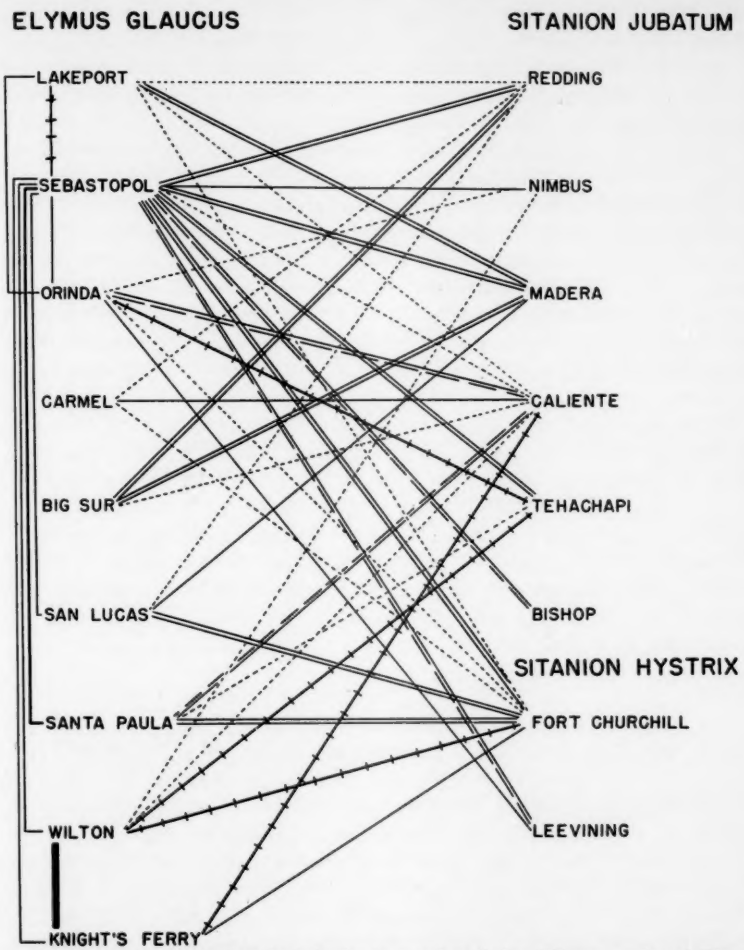


FIGURE 1.—Chart showing the behavior in crossing of nine different strains of *Elymus glaucus*, six of *Sitanion jubatum* and two of *Sitanion hystrix*. Thin broken lines, no hybrids obtained; cross marked lines, hybrids weak; thin continuous lines, hybrids vigorous, sterile, no allopolyploid obtained; double continuous lines, hybrids sterile, allopolyploid fertile; double line, one continuous, one broken, hybrids sterile, allopolyploid weak and largely sterile; thick continuous lines, hybrids vigorous and fertile.

glaucus can be termed a "good" parent, since it yielded vigorous hybrids in 7 out of 8 combinations attempted, while the Lakeport and Wilton strains are "poor" parents, yielding respectively 1 out of 4 and 0 out of 4 vigorous hybrids. In *S. jubatum*, the Madera strain is a "good" parent, while the Redding, Nimbus and Caliente strains are relatively poor. The other strains are

intermediate between these extremes. Again, there seems to be no clear relationship between the morphology or ecology of a strain and its behavior as a parent. The Sebastopol and Wilton strains are similar in appearance but behave very differently; the same is true of the Madera and Caliente strains of *S. jubatum.*

These results are less unexpected when one considers the relationship between the different "strains" of *E. glaucus* and probably also of *Sitanion.* SNYDER (1950) showed that most of the strains of *E. glaucus* collected along an altitudinal transect through the central Sierra Nevada are strongly isolated from each other by means of barriers of hybrid weakness or sterility, and the same is true of the strains discussed in the present work. As shown by figure 1, the only "interstrain" cross within *E. glaucus* which yielded fertile hybrids was Wilton × Knight's Ferry. It is interesting to note that the Knight's Ferry strain was found by Snyder to have the highest degree of interfertility with other strains of any strain studied by him; it formed fertile hybrids with three other strains. Since most of these "strains" behave toward each other like genetically distinct species, it is not surprising that they differ from each other in their compatibility with *Sitanion.* No hybridizations have been attempted between the different strains of *Sitanion,* but their appearance in the field resembles that of *E. glaucus;* in many instances two or three morphologically distinct types of either *S. jubatum, S. hystrix* or both species exist side by side without the presence of intermediates. It is very likely, therefore, that *Sitanion* contains a large number of reproductively isolated microspecies, just as does the *E. glaucus* complex.

MORPHOLOGY OF THE HYBRIDS

The external morphology of two different natural hybrids between *E. glaucus* and *S. jubatum* was described in an earlier paper (STEBBINS et al. 1946a). The artificial hybrids of this combination resemble these natural hybrids in all essential characteristics; the differences between them reflect clearly the differences between the parental strains involved. They are intermediate between their parents in all observable or measurable characters, and agree perfectly with the series of herbarium specimens identified as *Sitanion Hanseni.* The opinion previously expressed, that *S. Hanseni* is a collection of sterile hybrids between *E. glaucus* and either *S. jubatum* or *S. hystrix,* is therefore fully borne out by the results of artificial hybridization.

CYTOLOGY OF THE F_1 HYBRIDS

Chromosome behavior in a natural hybrid of *Elymus glaucus* × *Sitanion jubatum* was reported in an earlier paper (STEBBINS et al. 1946a). In this hybrid, meiosis in the majority of the sporocytes was nearly as regular as in the parental species. Chiasma frequency was somewhat lower than in the parents, and lagging chromosomes were frequently found.

For the present study, meiosis was examined in three of the F_1 hybrids, for which data on behavior at I metaphase are presented in table 2. One of them,

of the combination Sebastopol × Tehachapi (No. 005-2), was essentially similar to the natural hybrid from Tiburon reported earlier. The other two had more irregular meiosis. In Santa Paula × Caliente (009-3) and in Sebastopol × Leevining (006-1) the majority of the sporocytes had 13 bivalents and 2 univalents, while in both of these hybrids occasional larger configurations were observed. A chain of three chromosomes was found in one cell out of 28 in Santa Paula × Caliente (009-3), while a chain of four occurred in three cells out of 30 in Sebastopol × Leevining (006-1). In both of these hybrids, the number of sporocytes with lagging chromosomes and of tetrads with extra nuclei was somewhat larger than in the natural hybrid, as would be expected.

All of the F_1 hybrids produced nearly 100 percent of aborted pollen. While occasional grains were found which stained with aceto-carmine, their proportion was never more than a fraction of one percent. They also exhibited a high degree of sterility as to seed formation. In three separate experiments,

TABLE 2

Chromosome behavior at first metaphase of meiosis in F_1 hybrids of Elymus × Sitanion.

Hybrid	Mean and range of different configurations per PMC						No. of cells
	Multivalents		Bivalents		Univalents		
	Mean	Range	Mean	Range	Mean	Range	
Eg × SIj 005-2 (Sebastopol × Tehachapi)	0	0	13.56	12–14	0.88	0–4	43
Eg × SIj 009-3 (Santa Paula × Caliente)	0.04 (trivalents)	0–1	12.64	11–14	1.89	0–6	28
Eg × SIhx 006-1 (Sebastopol × Leevining)	0.10 (quadrivalents)	0–1	13.13	12–14	1.13	0–4	30

a very small number of viable seeds (about 1 per 10,000 florets) was obtained by interplanting F_1 hybrids with plants of the parental strain of *E. glaucus*. The results of these experiments will be presented in a later paper. For the present, the conclusion may be stated that the hybrids are almost completely sterile, but could on very rare occasions produce offspring through back crossing with one or the other parent, and still more rarely produce an F_2 individual.

MORPHOLOGY OF THE ALLOPOLYPLOIDS

In every progeny studied, the Elymus-Sitanion allopolyploids retained the condition intermediate between their parents which was characteristic of the F_1 hybrids. They showed the typical features of polyploids; taller, coarser stems, thicker leaves, and larger floral parts. Furthermore, little segregation was observed, at least in respect to those characteristics which most clearly differentiate the parental species. The "key character" differences between Elymus and Sitanion are the presence of a continuous rachis of the spike in Elymus, and a fragile, disarticulating rachis in Sitanion; and short, undivided

glumes in Elymus, while the glumes of Sitanion are elongate, awn-like, and mostly divided into two or more segments. Furthermore, *Elymus glaucus* is two to three times as tall as most strains of Sitanion; its culms and leaves are glabrous or slightly pubescent in most strains, while the majority of Sitanion strains are strongly pubescent; the spikelets of Sitanion usually have fewer florets than those of Elymus; their lemmas are smaller; and the palea is longer in relation to the lemma in Sitanion than in *E. glaucus*; and finally the lemma of Sitanion has two prominent lobes at the point of insertion of the awn, while these are completely absent in *E. glaucus*.

In floret number, glume length, lemma length, and length of lemma lobes, the C_2 progeny of Sebastopol-Tehachapi is somewhat more variable than the F_1 from which it was derived, and may be segregating to some extent. In all four of these characters, moreover, the Sebastopol-Tehachapi C_2 varies from the F_1 in the direction of more florets and greater size. Much of the variation, therefore, may be due to different degrees of expression of the increased size of parts often associated with polyploidy. The Sebastopol-Leevining C_2 progeny has the same type of variation in respect to lemma size, but varies toward shorter glumes, fewer florets, and shorter lobes than the F_1. This, associated with smaller size and increased sterility of the plants, may reflect the general weakness and internal disharmony of many plants of this progeny. In respect to number of divisions per glume, and in fact in respect to the general appearance of the glumes, the C_2 progenies show remarkably little evidence of segregation, and the same is true in respect to the relative length of lemma and palea. It appears, therefore, that the greater part of the segregation which does take place in these C_2 progenies is in respect to general characteristics of size and vigor; and affects relatively little the diagnostic characters which separate Elymus from Sitanion. Any observer would recognize all of the allopolyploid plants as similar to the F_1; none appeared in any of the cultures which in the sum total of its characteristics was anywhere near as close to either *E. glaucus* or Sitanion as it was to the F_1 plants. In short, these progenies behave genetically like typical allopolyploids, except for the appearance of weak, sterile plants in many of them.

This behavior would be expected were it not for the fact that the chromosomes of Elymus and Sitanion pair regularly with each other in the F_1 hybrids, and form a high proportion of multivalents in the allopolyploids, as is described below. Because of this type of pairing, segregation of Elymus vs. Sitanion characters might be expected. The possible reason for the absence of this type of segregation will be taken up in the discussion section.

CYTOLOGY OF THE ALLOPOLYPLOIDS

Because of the large number of chromosomes involved and the stickiness of the chromosomes, analysis of chromosome association in the allopolyploids was difficult, and only a relatively small number of sporocytes could be studied. Nine different plants from 4 different C_2 progenies were investigated. These were chosen to represent as wide a range of parental combinations as possible,

TABLE 3

Chromosome behavior at meiotic metaphase in Elymus-Sitanion allopolyploids.

	First metaphase Mean and range of different configurations per PMC										No. cells	% good pollen
	Quadrivalents		Trivalents		Total multivalents		Bivalents		Univalents			
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range		
Eg × SIj 1101-02	3.30	2–6	0.70	0–2	4.00	3–6	20.00	16–22	0.70	0–2	10	0.0
1101-11	4.44	2–6	0.47	0–2	5.21	3–7	18.00	14–22	0.47	0–2	15	63.0
1101-20	3.75	1–6	1.00	0–4	4.75	2–7	18.40	14–24	1.20	0–4	20	72.6
1103-14	3.82	1–7	1.58	0–4	5.40	2–7	16.58	13–23	2.88	0–10	17	31.0
1104-05	4.85	3–6	0.50	0–2	5.35	4–7	17.30	14–20	0.50	0–2	20	50.5
1104-38	4.04	2–7	0.90	0–2	4.94	2–7	17.05	12–21	3.19	0–12	21	42.0
1104-41	3.75	1–7	1.31	0–4	5.06	2–7	16.18	11–23	4.38	0–8	16	26.5
1104-51	3.70	2–6	1.15	0–3	4.85	3–6	17.70	14–22	1.35	0–4	20	9.6
Eg × SIhx 1105-23	4.55	1–7	0.85	0–3	5.40	1–7	16.80	14–26	1.65	0–5	20	

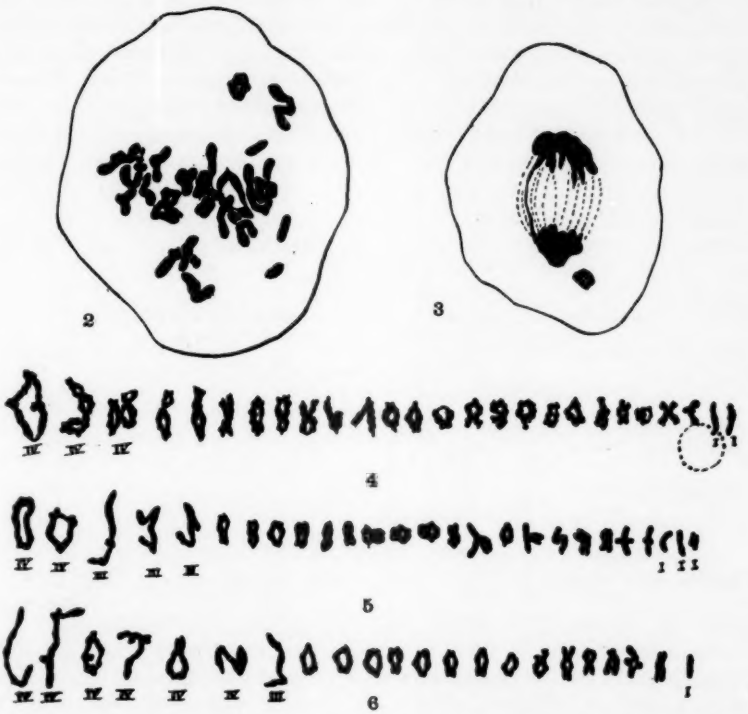
TABLE 4

Chromosome behavior at later stages of meiosis in Elymus-Sitanion allopolyploids.

	First anaphase			Second division			Quartets		% good pollen	% seed setting
	% regular divisions	% bridges	No. cells	% regular divisions	% bridges	No. cells	% regular	No. counted		
Eg × Slj 1101-02	30.1	....	83				53.8	65	0.0	20.6
1101-11	26.3	3.5	57	56.0	....	75	23.6	110	63.0	....
1101-20	39.0	....	131	38.3	....	120	39.5	114	72.6	43.2
1103-01	15.6	....	77	15.9	....	82	13.1	173	37.5	17.2
1103-06	25.5	2.9	102	40.3	1.5	67	26.4	110	43.3	25.8
1103-14	59.0	1.0	100	71.4	1.2	82	30.5	105	31.0	33.4
1103-19	32.5	0.8	123	68.3	1.6	63	78.6	103	4.5	1.4
1103-20	38.1	1.2	84	5.7	....	35	18.0	100	8.1	0.0
1104-05	42.9	0.8	128	41.2	5.9	68	73.3	105	50.5	20.2
1104-38	40.4	3.7	109	30.0	....	30	31.5	92	42.0	10.4
1104-41	40.2	....	97	58.5	....	106	64.7	102	26.5	1.1
1104-51	17.3	4.9	81	5.0	....	80	14.7	109	9.6	0.4
Eg × Slhx 1105-23	53.4	....	131	35.7	....	56	36.3	102	....	8.0

and within each progeny the plants investigated included those having the highest as well as the lowest degree of pollen and seed fertility found in the culture. The results are presented in tables 3 and 4.

As might be expected from the high amount of pairing found in the F_1 hybrids, multivalent configurations occur in all of the cells of every plant



FIGURES 2–5.—Drawings of meiosis in the allopolyploids of *Elymus glaucus–Sitanion jubatum*, no. 1101-20 (Sebastopol-Tehachapi), and 1104-51 (Santa Paula-Caliente, fig. 3 only). Fig. 2, a typical cell at I metaphase; fig. 3, chromatin bridge at late I anaphase; figs. 4-6, complete chromosome complements drawn out separately from 3 cells at late prophase, showing the nature of the chromosome configurations. All figures from aceto-carmine smears, reproduced at a magnification of 1500 ×.

investigated. Furthermore, the mean number of multivalents is nearly the same in all of them. With the exception of that for plant 1101-02, the individual plant means are all included in the narrow range of 4.75 to 5.40 multivalents per sporocyte. While the number of cells examined is too small for adequate statistical comparison, the fact that in many plants the number of multivalents per sporocyte may range from 1 to 7 indicates that the differences between means are largely if not entirely nonsignificant. The multivalents

consist of both trivalents and quadrivalents, which are mostly of the ring or chain type with non-terminalized chiasmata (figs. 4–6). It is an interesting fact, which will be discussed below, that the number of multivalents per sporocyte never exceeds 7, and that this is the maximum number in 7 out of the 9 plants investigated.

Unpaired univalents occurred in some sporocytes of all of the plants investigated. Unlike those of multivalents, however, the mean numbers of univalents vary greatly from plant to plant. In the three plants of culture No. 1101 and in plant 1104-05, the mean number of univalents nearly or quite equals the mean number of trivalents. This suggests that in these plants univalents occur almost entirely because of mechanical difficulties involved in multivalent association, and that the univalent chromosomes present are members of potential quadrivalents which failed to form chiasmata with their homologues. The same factor accounts for some of the univalents in all of the plants investigated, but in such plants as 1104-38 and 1104-41, in which the

TABLE 5

Distribution of percentage values for pollen and seed fertility in four C_2 progenies of Elymus-Sitanion.

	Number of plants with fertility percentages of							
	0–10	10.1–20	20.1–30	30.1–40	40.1–50	50.1–60	60.1–70	70.1–80
Eg × Slj 1101 Pollen	3	1	0	3	1	4	9	8
Seed	3	0	4	3	3	5	1	1
Eg × Slj 1103 Pollen	8	5	3	21	13	7		
Seed	14	12	12	6	3	2		
Eg × Slj 1104 Pollen	7	9	12	8	6	3		
Seed	16	12	9	8	1			
Eg × Slhx 1105 Pollen	2	2	11	9	10	10	2	
Seed	25	17	3					

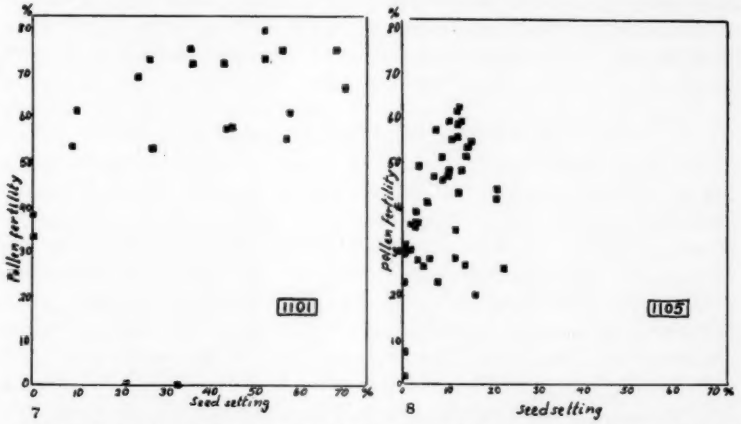
number of univalents is 3–4 times that of trivalents, the failure of chromosomes to pair must be due partly to other causes. It will be noted that both of these plants are descended from the F_1 hybrid culture 009, which had a higher frequency of unpaired univalents than any other F_1 investigated. It is also interesting to note that three of the four plants with a minimal number of univalents belong to culture No. 1101, which is descended from an F_1 hybrid, No. 005-2, which had nearly normal meiosis; and that the fourth of these allopolyploids with few univalents was the most pollen fertile of the 1104 culture. Hence there appears to be a relationship between high frequency of univalents in the F_1 hybrids and in the allopolyploids, and a less clear relationship between infrequency of univalents and high pollen fertility.

Irregularities at later stages of meiosis consist mostly of lagging chromosomes, which often form extra nuclei or extra cells in the tetrads. The frequency of these irregularities may be slightly correlated with the frequency of univalents at I metaphase, but there are plants with few univalents and irregular later stages (No. 1104–51) and others with many univalents and

comparatively regular later stages (No. 1103–14). It appears as if the disturbances of meiosis in these allopolyploids are caused by a variety of factors, acting at different stages in the process. Bridge-fragment configurations are uncommon, and no more frequent than in the F_1 hybrids.

POLLEN AND SEED FERTILITY

As can be seen from table 5, the various C_2 progenies segregated widely for pollen and seed fertility. Furthermore, there does not seem to be any clear relationship between percent of stainable pollen and percent of florets which produce viable seed, either between cultures or between the different plants of the same culture. Of the four cultures investigated most carefully, No. 1101 (Sebastopol-Tehachapi) contains a relatively large number of plants which



FIGURES 7 AND 8.—Scatter diagrams showing the relation between pollen fertility (percentage of grains well stained with acetocarmine and bearing 3 nuclei) and seed fertility (percentage of florets bearing well-developed seeds) among plants of two C_2 allopolyploid progenies, nos. 1101 (Sebastopol-Tehachapi) and 1105 (Sebastopol-Leevining).

are reasonably fertile as to both pollen and seed; No. 1103 (Big Sur–Redding) is intermediate in pollen and relatively low in seed fertility; No. 1104 (Santa Paula–Caliente) is intermediate in pollen and low in seed fertility; while No. 1105 (Sebastopol–Leevining) is very variable in pollen and very low in seed fertility.

Figures 7 and 8 are scatter diagrams showing the distribution of the individual plants in cultures Nos. 1101 and 1105 as to pollen and seed fertility. The lack of correlation between these two types of fertility is evident from these diagrams; it is equally evident from the individual data of cultures Nos. 1103 and 1104, and is probably a general phenomenon. It is evident that the observed fertilities depend upon a number of factors, and that those which contribute to seed sterility are at least in part different from those causing abortion of pollen.

Seed set is influenced to a greater degree by environmental conditions than is pollen fertility. This was particularly evident in the two more sterile cultures, Nos. 1104 and 1105. In culture No. 1104, for example, a single plant (No. 50) gave the following percentages of seed setting in 11 different heads, maturing at different times: 29.6, 22.8, 22.2, 19.8, 16.1, 13.8, 12.8, 9.8, 7.1, 4.7, 4.5. Similar results were obtained for several other plants of both cultures; whereas many plants were relatively uniform in this respect. In general, however, the differences between plant means were significantly greater than the variation within a plant, so that the data given on table 5 and figures 7 and 8 are actually meaningful. A further investigation of the causes of seed sterility is being made by Mr. Rui D'Cruz; his preliminary results indicate that even in the more sterile plants a high percentage of embryo sacs develop to maturity. Most of the factors affecting seed development act after the time of pollination, and affect the processes of fertilization, embryo, and endosperm development. It is not surprising, therefore, that they are different from the factors which condition the abortion of pollen.

DISCUSSION

Nature of segregation in the allopolyploids.—In the descriptive section, emphasis was placed upon the small amount of detectable segregation in respect to those characters which most sharply differentiate the parental species; height, pubescence, fragility of rachis; glume shape and awn length. This retention of intermediacy would be expected in a typical or genomic allopolyploid, but in segmental allopolyploids, like the present ones, in which multivalent association occurs regularly, one would expect some degree of segregation in respect to the parental characteristics. This has been observed by JAKOB (1953) in the segmental allopolyploid of *Bromus ciliatus-laevipes,* by OWNBEY (1950) in Tragopogon, and by OKA (unpubl. data, in litt.) in polyploids derived from the partly sterile "intersubspecific" hybrids of cultivated rice. GAJEWSKI (1953) found in the amphidiploid *Geum macrophyllum-rivale* a situation essentially similar to that in the present allopolyploids; i.e., pronounced segregation for fertility but little or no segregation for the morphological characteristics which differentiate the parental species. In his example, however, this type of behavior would be expected on the basis of chromosome behavior, since no multivalents were observed at meiosis.

The present situation might best be explained by making the following assumptions concerning the genomic relationships between *Elymus glaucus* and Sitanion spp. The 1 to 7 multivalents found in the allopolyploids represent for the most part random association between chromosomes belonging to one genome each of *E. glaucus* and *S. jubatum* or *S. hystrix.* These genomes are completely homologous, and do not carry the genes responsible for the principal differences between the species concerned in external appearance. On the other hand, the second genome of *E. glaucus* is more strongly differentiated from the corresponding genome of *Sitanion* so that the chromosomes of these two genomes, while able to pair to a certain extent in the F_1 hybrid, do not

become associated in the allopolyploid, each chromosome pairing preferentially with its exact duplicate. In short, the allopolyploids concerned may be autopolyploid with respect to four of their eight genomes, and segmentally allopolyploid with respect to the other four. In terms of genome formulae, that of *E. glaucus* was designated by STEBBINS and SINGH (1950) as $A_2A_2E_1E_1$, and that of Sitanion could be designated as $A_2A_2E_sE_s$, the designation E_s being given to an *E* genome which bears the distinctive morphological characteristics of Sitanion. The present allopolyploids would therefore become $A_2A_2A_2A_2E_1E_1E_sE_s$. Their multivalents would involve chiefly or entirely the A_2 genomes, and the small amount of segregation would be due to the rarity of pairing in the allopolyploids between chromosomes of the E_1 and E_s genomes.

If this were true, we should have to assume that the A_2 genomes of *E. glaucus* and Sitanion, although not sufficiently differentiated to prevent multivalent association, nevertheless are differentiated in respect to genes or small chromosome segments which affect vigor and fertility. This would be the only basis for explaining the wide segregation for these characteristics in all of the C_2 progenies. This question will be considered further in the next section of the discussion.

Nature of the sterility in the F_1 hybrids and allopolyploids. In the first paper of this series (STEBBINS et al. 1946), the sterility of the F_1 hybrid between *Elymus glaucus* and *Sitanion jubatum* was considered to be chromosomal, and to be caused by differences between the parental species with respect to small rearrangements of chromosomal material, which were termed cryptic structural differences between the parental chromosomes, since they could not be detected either on the basis of visible differences in chromosome morphology or by the nature of chromosome association in the F_1 hybrid. The condition in this hybrid was described as cryptic structural hybridity. In a later publication (STEBBINS 1950, pp. 220–227) the senior author cited more examples of this phenomenon, and suggested some specific ways by which it could occur. He also suggested that chromosomal sterility due to cryptic structural hybridity could in many examples be combined with genic sterility, and that the two processes could be effective to varying degrees.

The senior author, however, was not the first to recognize the existence of sterility due to cryptic structural hybridity, since its presence was indicated in hybrids of Gossypium by STEPHENS (1945), and was more fully discussed later (STEPHENS 1950). STEPHENS pointed out clearly the great difficulty in distinguishing between genic and small chromosomal differences, and suggested genetic tests for differentiating between them.

At present, therefore, the existence of cryptic structural differences as a cause of hybrid sterility seems to be reasonably well established. There is, however, one difficulty in explaining on the basis of this phenomenon many examples of hybrid sterility, including that between *Elymus glaucus* and Sitanion spp. This is the absence or rarity of major structural differences between the species concerned. One might expect that chromosomal aberra-

tions involving segments of all sizes would arise with about equal frequency, and therefore that cryptic structural differences would always be accompanied by major, detectable difference in chromosomal arrangement.

The rarity or absence of major structural differences could be explained in either of two ways. In the first place, the incidence of rearrangements of small chromosomal segments may be higher than that of large ones. This condition, if true, would be hard to detect in most or all of the experimental material in which chromosome aberrations have been observed, because of the difficulty of recognizing small rearrangements. There is, however, very suggestive evidence in the observations of McCLINTOCK (1951) on the behavior of the *Ds* and *Ac* loci in certain genetic stocks of maize. The relatively frequent transposition of individual loci from one part of the chromosome complement to another has here been detected because of the presence of a visible breakage-fusion-bridge cycle which initiated the changes, and of genetically marked stocks which permitted their recognition. It seems not improbable that similar changes would occasionally take place in other plant species and pass unrecognized for lack of any means of detecting them.

Further evidence that small alterations of chromosome structure may occur in some plants with a frequency previously unexpected is provided by the observations of LIMA-DE-FARIA (1952) on pachytene chromosomes of rye. He noted that the form studied by him was heterozygous not only for certain differences in chromomere size which appeared constantly in every cell studied, but also for a structural rearrangement in which the number of chromomeres involved varied from one pollen mother cell to another of the same anther. This suggests that small rearrangements of chromosomal material had been taking place during the growth of the anther involved.

The second explanation for the presence of only small structural differences between the chromosomes of two related species would be on the assumption that one of the two species is secondarily derived through introgression from hybrids between the other and some distantly related species. SNYDER (1951) suggested that many of the microspecies or genetically isolated entities which exist within the taxonomic species *Elymus glaucus* could have arisen in this fashion, and more recent evidence of the senior author (unpubl.) supports this hypothesis, as well as the hypothesis that still others of these microspecies have arisen as introgressants from hybrids between *E. glaucus* and *Agropyron Parishii*. The basis of the concept of introgressive hybridization (ANDERSON 1949, 1953) is that backcross derivatives containing nearly all of the chromosomal material from one species but a few blocks of genes from another species are more likely than any other products of interspecific hybridization to be both viable and fertile, as well as adapted to some available ecological niche. If the original hybridization is between two species which are so distantly related to each other that their chromosomes are largely non-homologous, then a stabilized derivative of introgression may differ from its recurrent parent in respect to several small chromosome segments derived from the second original parent. In self fertilizing plants, such introgressants may be ex-

pected to become homozygous for such allogenous segments, and therefore fully fertile. But when outcrossed to their recurrent parent, they will produce F_1 hybrids heterozygous for these allogenous segments, and therefore partly sterile in spite of the essentially normal meiotic behavior due to the homology of the majority of the chromosomal complement. On the basis of this hypothesis, one would expect to find the largest number of examples of cryptic structural differences to exist between species capable of self-fertilization, a fact which is borne out by the known examples of this phenomenon, including those in the tribe Hordeae. In the species complex discussed in this paper, therefore, the presence of many small structural differences and relatively fewer (though definitely several) larger ones is not hard to explain, and best accounts for the greater part of the sterility in the F_1 hybrids.

That genic sterility exists also is suggested by the high degree of sterility and asynapsis in some of the allopolyploids, particularly those involving the Caliente strain of *Sitanion jubatum*. It is perhaps not an accident that the greatest difficulty in producing allopolyploids, and the highest degree of sterility of these allopolyploids when produced was found in combinations involving strains which were "poor" parents; i.e., which were difficult to hybridize. The stage at which development was arrested in the unsuccessful crosses is not known, but may very likely have been at some time during the development of the embryo, as is true in other unsuccessful or difficult hybrid combinations in the Hordeae (BRINK, COOPER and AUSHERMAN 1944; BEAUDRY 1951). One may postulate, therefore, that certain gene combinations will function well enough to produce growth at all times except one or two critical stages of development, when the organism is particularly sensitive to genic disharmony, and cell divisions are very likely to become disturbed. One of these critical stages is evidently in the early mitotic divisions of the embryo and particularly the endosperm, while the other is during meiosis. Failure of embryo development and genic sterility at meiosis may therefore represent different degrees of expression of the same kind of genic disharmony.

Relation between degree of affinity between the parents and success of the allopolyploid.—Some authors, particularly CLAUSEN, KECK and HIESEY (1945, p. 69) have maintained that an allopolyploid ("amphiploid") is most likely to succeed if its parent species are so distantly related that they are classified in different cenospecies, and interchange of genes between them is impossible on the diploid level. This is probably true in a statistical sense; i.e., the proportion of vigorous F_1 hybrids which yield vigorous, fertile allopolyploids probably increases as the parental species become more distantly related to each other. But the number of exceptions to this generalization is increasing as more and more artificial and natural allopolyploids become known. Several examples of successful allopolyploids between closely related species may now be added to those cited by STEBBINS (1950). Among the most notable are those of Tragopogon (OWNBEY 1950), Oryza (CUA 1952) and Geum (GAJEWSKI 1953), as well as those described in the present paper. Furthermore, the present evidence indicates that within a series of closely re-

lated forms, the degree of affinity between the two parental species bears little or no relationship to the success of the allopolyploid. The factors which make for the success or failure of an allopolyploid are so numerous and complex in their interactions that no generalizations are accurate enough to serve as a reliable guide to either the student of evolution or the plant breeder who is attempting to produce new and valuable allopolyploid combinations. At present, success in this field is most likely to come from patient trial and error involving many hybrid combinations of all sorts, and an increasingly greater familiarity with the species complex under study.

SUMMARY

Artificial hybridization is reported between nine different strains of *Elymus glaucus* and six strains of *Sitanion jubatum* as well as two strains of *S. hystrix*. The degree of crossability varied greatly with the combination concerned, since many crosses failed altogether, others produced only weak F_1 hybrids, while still others produced vigorous but highly sterile hybrids. Some strains of all three species produce vigorous hybrids in a relatively large number of combinations, while others rarely produce them. The F_1 hybrids are intermediate between their parents in visible morphological characteristics, and resemble the natural hybrids which have been classified by systematists as *Sitanion Hanseni*. They have nearly regular meiosis, although in some of them univalent chromosomes were regularly observed.

The allopolyploids of the C_2 generation, descended from chromosomal chimaeras produced by colchicine treatment of F_1 hybrids, retain the intermediate condition of the F_1 in morphological characteristics but are larger and coarser in their floral parts. Some progenies were relatively homogeneous in size and vigor, while in others many small, weak plants appeared. There was segregation for high vs. low pollen and seed fertility in all progenies, but this segregation was most marked in those progenies which included relatively many weak plants. Within any one progeny, there was little or no correlation between the percentage of stainable pollen and the percentage of florets which produced viable seed.

Meiosis in the allopolyploids was more irregular than in the F_1 hybrids. In all of them there appeared from 1 to 7 trivalents and quadrivalents, the mean number of multivalents ranging from 4.00 to 5.40. Since non-significant differences in multivalent frequency were found between plants which differed greatly in fertility, the formation and segregation of multivalents probably has little to do with fertility in these allopolyploids. Univalent frequency at metaphase, likewise, was not greater in those plants with low fertility. On the other hand, the plants with high percentages of aborted pollen tended to have a relatively high percentage of first and second anaphases with lagging chromosomes, and of sporads with extra nuclei.

The small degree of segregation for morphological characters in the allopolyploids in spite of the regular presence of multivalents at meiosis is attributed to an unusual combination of auto- and allopolyploidy, and genome

formulae are suggested which would explain the situation. Additional evidence is presented to support the hypothesis that the sterility in the F_1 hybrids is largely chromosomal, and due to cryptic structural hybridity. Genic sterility is probably also present to a varying degree, and appears to be associated with the incompatibility in species crosses possessed by some of the parental strains. The authors believe that no reliable generalizations may yet be made by which the investigator can predict the success or failure of a particular allopolyploid combination.

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EVIDENCE FOR EPISTATIC ACTION OF GENES FOR ANTIGENIC SUBSTANCES IN SHEEP[1]

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PREVIOUS studies on the blood of sheep (ANDERSEN 1938; YCAS 1949) have shown the presence on the erythrocytes of an antigenic character, called R, demonstrable by a normally occurring antibody present in the sera of some sheep lacking the R substance. The report by YCAS suggested that the R character was inherited as a dominant in contrast to its absence. Further investigations by STORMONT (1951) showed that sheep bloods, which failed to react with anti-R reagents (i.e., typing fluids specific for the R-substance), might react with normal sera of certain cattle which had been absorbed with sheep cells of group R. This reagent obtained from cattle serum was called anti-O. In tests of over 100 sheep bloods with both anti-R and this new reagent (anti-O), the reactions were mutually exclusive. Since the new reagent was not reactive with the cells of heterozygotes, STORMONT suggested that the genes determining the R and O characters were allelic, the gene for R being dominant. However, in this paper the O character will be called " r " in order to have the terminology in concordance with genetical practice.

This report describes a new group " i " of sheep erythrocytes. Cells are classified as belonging to group i if they fail to react with both anti-R and anti-r sera. Further, this report will deal with the serological characteristics and the inheritance of the characters R, r and i.

MATERIALS AND METHODS

The study has been performed on about 400 individual sheep belonging to the University of Wisconsin, of the following breeds: Hampshire (111), Southdown (67), Shropshire (91) and mixed breeds (130).

Blood was taken from the jugular vein into a clean glass tube containing isotonic citrate solution (2 percent sodium citrate and 0.5 percent sodium-chloride), when the sample was used for a cell suspension. If serum was desired, citrate was not used, and the serum was permitted to clot. After centrifugation the serum was drawn off and stored. The sera were not heated to destroy complement, since it was observed that unheated sera did not contain sufficient complement activity to interfere with the results.

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Sera from different R negative and r positive (group r) individuals were used as testing fluids (reagents) for the R character. Dr. C. Stormont, of the School of Veterinary Medicine, Davis, California, kindly furnished normal serum from one cow, from which anti-r reagents could be produced by absorption with R cells. In general, absorptions performed in the course of these investigations were carried out as follows: 1 volume of appropriately diluted serum was added to 1 volume of washed, packed erythrocytes. The mixture was shaken and allowed to incubate at room temperature ($24° \pm 2°C$) for one-half hour. The tubes were centrifuged, the supernatant serum removed and placed in a tube containing an equal volume of freshly washed erythrocytes, shaken and then allowed to incubate for 30 minutes at room temperature. After another half hour in the refrigerator ($2° \pm 2°$), the tubes were centrifuged and the serum was collected.

The technique involved in the lytic test has been described elsewhere (Stormont and Cumley 1943; Ycas 1949). Briefly, 0.1 ml of diluted anti-serum and 0.05 ml of a 2.2–2.5 percent cell suspension were mixed in a test tube. After 15 minutes incubation at room temperature, 0.05 ml of complement, diluted 1 : 2, was added. Rabbit serum, which was used as complement, usually contained species antibodies for sheep cells. Hence, it was absorbed with pooled sheep cells before it was used as complement. (The absorption for complement was performed by mixing equal volumes of fresh, undiluted rabbit serum and packed, washed sheep cells for ten minutes in a tube surrounded by crushed ice.) The tubes containing antiserum, cells and complement were shaken and allowed to incubate at room temperature for one-half hour, after which they were again shaken. The test was read after an additional one and one-half to two hours incubation and checked after still another one and one-half hours incubation.

The different antisera were ordinarily used in a dilution of 1 : 4, which was more than sufficient to give complete lysis, if the antigenic factor was present on the test cells. However, the cells of young lambs were tested at a serum dilution of 1 : 2. All cells which failed to react with either anti-R or anti-r sera were re-tested at a dilution of 1 : 2 of both antisera.

Inhibition tests were performed to estimate the occurrence and the relative concentration of soluble antigenic substances in the sera of different individuals. If a serum contains serologically active substances identical with or related to substances on the cells, then the homologous antiserum, if mixed with sera containing the soluble substance, will lose its ability to lyse the homologous cells. The serum whose inhibiting activity is to be measured is called the "inhibitant." This is diluted in doubling dilutions. The highest dilution inhibiting the reaction between the antiserum and the test cells will give an approximate measure of the amount of the soluble substance present in the serum. The inhibition test was performed as follows: 0.05 ml of the inhibitant and 0.1 ml of the antiserum (reagent) were mixed in a test tube and permitted to stand for one-half hour, after which the appropriate blood cells (which usually were lysed completely by the reagent) and the comple-

ment were added in that order. Controls were included to test for antibodies in the inhibitant against the test cells.

STORMONT (1949) and STONE (1953) have reported that it was possible to convert erythrocytes of cattle from J negative into J positive by treating them with serum from J positive individuals which contained the soluble, serologically active J substance. In order to determine if it was possible to " coat " sheep erythrocytes of one type with the soluble substance of another type, " coating experiments " were performed. The cells of one group were mixed with serum of another group in the proportion 1 : 14 and allowed to stand in a water bath at 37°C for 5 hours, after which they were placed in a refrigerator overnight. If the cells were not coated after that time, they were removed to fresh serum and incubated again for 5 hours at 37°C. When in the water bath the tubes were shaken once each hour.

EXPERIMENTAL RESULTS

Previous studies by YCAS (1949) and STORMONT (1951), and the present studies indicate that sheep bloods may be classified into groups as outlined below:

R-substance	r-substance	Anti-R	Terminology of STORMONT	Proposed terminology
+	0	0	R	R
0	+	+	O	r
0	0	+ or 0		i

R indicates the presence of the R substance on the cells and in the serum; r indicates the presence of the r substance on the cells and in the serum, which also contains anti-R; i refers to a group lacking R and r substances, and anti-R is present in the serum of only some of the individuals. The i group was not detected prior to this report.

Of the 399 bloods tested, 222 were classified as R, 151 as r, and 26 as i (16 adults and 10 lambs) since their cells reacted neither with anti-R nor with anti-r sera. Since lambs normally develop the R or the r substance on their cells before they reach an age of 50 days, and those classified as i ranged in age from 80–129 days, it seems very unlikely that the absence of both R and r on the cells of these 26 animals is due to a delayed appearance of either R or r.

YCAS (1949) demonstrated by inhibition tests that sera from individuals classified as group R contained a soluble substance, which inhibited the reactions between anti-R sera and R cells. Similar results were observed by NIEMANN SORENSEN, RENDEL and STONE (1954) for the sera of group r, since such sera inhibited the reactions between the r-reagents and r cells. These observations indicated that sera of group r contained a soluble substance identical or closely related to the antigenic substance of the r cells.

In this study 20 r individuals were tested for the presence of R antibodies in their sera. Thirteen sera were found to contain strong anti-R, while 7 had

only weak antibodies, which presumably were anti-R. This conclusion is based on the fact that these sera did not lyse any cells of group r, but reacted with R cells which gave strong lysis with other anti-R sera, and failed to react with cells giving weaker reactions with these strong anti-R sera. However, the possibility that these reactions were due to other normal antibodies cannot be ruled out entirely.

The sera of 14 adults and 4 lambs of group i have been tested for substances inhibiting anti-R and anti-r sera. For each of these antisera not only i serum, but also, as controls, R and r sera were used as inhibitants. At first the tests were difficult to interpret, since it was found that the i serum consistently inhibited the anti-R activity of r serum one or two dilutions more than did the control r sera, while i sera and R sera inhibited anti-r approximately equally. Consequently the inhibiting activity of i and R serum on anti-r serum should be considered as non-specific. The fact that i serum inhibited anti-R sera more strongly than did the r serum could be interpreted as meaning that the sera of individuals classified as i may contain a small amount of the R substance, in a concentration too low to be acquired by the cells but great enough to cause observable inhibition of anti-R sera. This finding is similar to certain observations by STONE (1953) in studies on the J-substance of cattle. However the results might also be interpreted as nonspecific inhibition, since r sera used in the inhibition test with anti-R had previously been absorbed with R cells to remove anti-R and still showed a slight inhibition of the reaction between R cells and R-antisera. This difference in inhibiting capacity between the r (anti-R) and i sera might be accounted for by assuming that the absorption of the r sera by R cells decreased their normal and possibly non-specific inhibiting capacity.

To investigate the question further, five sera from i individuals, three from R and three from r, were absorbed by pooled cells of group R and r. Cells of type r were included to absorb the reactions from other normal antibodies, which might occur in any of these sera. The same types of cells were used for absorption and as test cells. Two anti-R sera were included. One of these anti-sera was used at dilutions of 1/256 and 1/128 (containing 4 and 8 units of hemolysin, respectively,) the other was used at dilutions of 1/128 and 1/32 (containing 4 and 16 units of hemolysin). (The highest dilution of a reagent giving definite hemolysis is taken to contain one unit of hemolysin.) The anti-r serum had 8 and 4 units of hemolysin in its different dilutions. In table 1, the results of the inhibition tests are shown for one representative serum from each of the three groups of inhabitants. It may be seen that the serum from an individual of group i does not inhibit the R-antibodies more than the non-specific inhibition of the r-serum. Also there was no significant difference between i and R sera in their inhibiting action of anti-r sera.

In addition, cells from 10 more sheep of group i have been used in absorptions of anti-R and anti-r sera. The specific antibodies in the respective antisera were not removed. Thus the conclusion seems justified that sheep of group i do not contain either R or r substances on their cells or in their sera.

The question arises as to whether the sera of group i contain R- or r-antibodies. The sera of 23 individuals of group i (14 adults and 9 lambs with an average age of 117 days) have been tested for R- and r-antibodies. None of these sera were found to have anti-r, neither did any of the i sera from adults contain R-antibodies. Two of the sera from lambs showed strong or intermediate R-antibodies, while 7 lacked anti-R. One of these latter had strong normal antibodies, which did not parallel either anti-R or anti-r.

The marked variation in the antibody titer of anti-R normal sera reported by YCAS (1949) suggests the possibility that some individuals of group i, which at a certain time lack R-antibodies might at another time contain them. However, nine of the adult individuals of group i have been tested in two succeeding months and two individuals have been tested five times over a period

TABLE 1

The inhibition of anti-R and anti-r sera with sera of the groups, R, r and i.

Inhibitant		R-serum				r-serum				i-serum			
Reagent		Anti-R		Anti-r		Anti-R		Anti-r		Anti-R		Anti-r	
Units of hemolysin		8	4	8	4	8	4	8	4	8	4	8	4
Dilution of inhabitant	1/1	0	0	4	0	4	1	0	0	4	tr	4	3
	1/2	0	0	4	3+	4	3	0	0	4	3	4	4
	1/4	0	0	4	4	4	3+	0	0	4	4	4	4
	1/8	0	0	4	4	4	4	0	0	4	4	4	4
	1/16	0	0	4	4	4	4	0	0	4	4	4	4
	1/32	3	0	4	4	4	4	4	0	4	4	4	4
	1/64	4	0	4	4	4	4	4	0	4	4	4	4
	1/128	4	1	4	4	4	4	4	0	4	4	4	4
	1/256	4	2	4	4	4	4	4	0	4	4	4	4
Control (no inhibitant)		4	4	4	4	4	4	4	4	4	4	4	4

Symbols: 4 = complete hemolysis; 3 = hemolysis not quite complete; 2 and 1 = incomplete or intermediate hemolysis; Tr = weak hemolysis; 0 = no visible hemolysis.

of 11 months (once in June 1952 and in February, March, April and May, 1953) and no antibodies against either R or r cells have been found. Thus it seems justified to conclude that some individuals of group i lack R-antibodies and that presumably all individuals of group i lack r-antibodies. A tentative genetical interpretation of the findings that some individuals of group i have anti-R, while others lack it, will be discussed later.

YCAS (1949) found that the R substance was present at birth in the serum but not on the cells of R lambs. The substance was presumably acquired by the cells from the serum, at an average age of three weeks. The results of the present study agree with those of YCAS on the R character and show in addition that the r substance was not present on the erythrocytes of newborn lambs.

Among the cells of a group of 78 lambs of an age varying from 9 to 34 days, 8 were of group R and 16 of group r, while the remaining 54 were completely negative or showed only weak reactions with a specific reagent. (A

reaction was designated as positive only if half or more of the cells were lysed.) The youngest age at which the lambs could be shown to belong to group R or r was 19 and 20 days, respectively. It was found that the majority of the 54 " double negatives " developed the R and r factors on their cells at an age of about one month. Furthermore, inhibition tests with the sera of four lambs showed that the r substance was present in the serum of lambs of group r before it was detectable on the cells.

It can be concluded that there are not two, as previously believed, but three distinct types of sheep blood: (1) R, characterized by the R-factor on the cells and the soluble R substance in the serum; (2) r, having the r factor on the cells and the soluble r substance and R-antibodies of varying strength in the serum; (3) i, having neither R nor r on the cells or in the serum. Some individuals of this group have demonstrable R-antibodies in their sera.

Coating experiments

To investigate if it was possible for the erythrocytes of sheep of one type to acquire the soluble substance of another type, coating experiments were performed by the method described earlier. When cells of groups r and i were treated with sera from individuals of group R a significant coating was observed, after the cells had been treated 5 hours at 37°C and 15 hours in the refrigerator and then tested with a reagent for R. After repeated incubation of the cells in fresh serum the strength of the subsequent lytic reactions increased and in some cases complete lysis was observed. Thus it was possible to change r cells into those reacting with both anti-R and anti-r sera, and to convert i cells into R-cells. The r cells coated with R substance behaved as expected of heterozygotes, such as the AB or MN of man, or the $F^{V}F^{V}$ of cattle, in that they were reactive with the reagents for both contrasting antigenic characters. However, STORMONT (1951) has reported, and our findings are in agreement, that the cells of heterozygotes (R/r) are not reactive with the reagent for r.

Some variability was observed in the ability of different sera to coat the cells of an individual, and also in the ability of different cells to be coated. (Comparable variability was also noted by STONE (1953) in experiments on J in cattle.) When r sera were used for coating of i cells only insignificant coating was observed after the first treatment (5 hours at 37°C and 15 hours in refrigerator). After prolonged treatment, however, it was possible to convert i cells into cells of group r. Thus it should be theoretically possible to change cells of group i into cells reacting with both anti-R and anti-r sera, if first treated with r and *later* with R sera, because r sera seem invariably to contain antibodies against R. On the other hand, it has not been possible to coat R cells with r substance, because we have never been able to exhaust the r sera of R-antibodies and R cells were therefore lysed during the coating treatment. However, these results indicate that both the R or r soluble substances, present in sheep serum of a particular group, are acquired by the erythrocytes from the surrounding serum.

Genetical studies

This study includes 148 matings, divided into 9 sire-families. Five sires were of group R, three of group r, and one of group i. The numbers and blood types of the offspring from the different matings are summarized in table 2. The matings of R × R individuals gave 30 offspring with R, 5 with r, and 2 with i. From r × r matings only r progeny (44) were obtained, and in matings R × r and r × R there was a slight excess of R in comparison with r individuals (55 : 35). These results would be expected, if R is dominant to r. Thus these data agree with the proposal of STORMONT (1951), that the gene determining the group R is dominant over that determining the r substance.

From the data summarized in table 2, it may be seen that offspring of type i appeared in the offspring of matings of R × R and R × r. And an exception to the usual rule within all species that an individual carries a cellular antigen

TABLE 2

The numbers and classes of offspring from different matings.

Blood type of parents		No. of sires	No. of matings	Offspring with blood type			Total No. of offspring
sire	dam			R	r	i	
R	R	5	29	30	5	2	37
R	r	5	12	12	1	2	15
r	R	3	52	43	34	0	77
R	i	1	1	1	0	0	1
i	R	1	11	7	2	8	17
r	r	3	28	0	44	0	44
r	i[1]	2	6	7	1	0	8
i	r	1	7	4[2]	3	2	9
i	i	1	2	0	0	2	2
Total			148	104	90	16	210

[1]All parents and offspring in this kind of mating have been tested at least twice, some three or four times, with anti-R (sheep) and anti-r (cattle) sera.
[2]Two of these with R were adults.

only if either or both parents also possess it may be noted in that progeny of group R appeared from the reciprocal matings of r × i. Hence it appears that the i group behaves in inheritance as a recessive, but definitely not as a recessive to either *R* or *r*. For further critical analysis, the blood types of the individuals involved in pertinent matings of these three rams are therefore given in table 3. It may be deduced from table 3 that ram 468 is *R/r*, on the assumption that *R* is dominant to *r*, since from two matings (1 and 5) to ewes with R, both R (B41) and r (B40, 228) offspring were obtained. However, two offspring of group i (252, 287) were sired by ram 468, one from a mating (8) to a ewe of group r, the other from a mating (11) with a ewe of group R. Parallel results are shown in the three matings (86, 88, 89) given for ram 876 (group R) in that this ram sired both R and r lambs from mating to a ewe with r, an r lamb from a ewe with R and an i lamb from a ewe with R. Further, the two offspring with i (numbers 252 and 287), from sire 468,

in matings (17, 19) to a ram with r (158987) produced a lamb with R (mating 17) and lambs with R and r, respectively. The ewe 252 with blood type i must have carried the gene for R which was expressed in her offspring B5. The data of table 2 are also pertinent in which the ram of blood type i, in matings to ewes with R, produced 7 offspring with R, 2 with r, and 8 with i. The data in tables 2 and 3 are consistent with the assumption that the genes *R* and *r* produce their respective products only when the dominant allele of *i* at another locus is present.

Additional evidence of the inheritance of the groups R, r and i is given in the pedigrees of figures 1 and 2, in which the phenotypes and probable genotypes are pictured for individuals representing three generations. From the data of table 3, it appears that the genotype of ram 468 is *I/i R/r*, that of ewe

TABLE 3

Selected data from three sire families.

Mating number	Sire		Dam		Offspring	
	No.	Bloodgroup	No.	Bloodgroup	No.	Bloodgroup
1	468	R	257	R	B40	r
					B41	R
5	468	R	338	R	228	r
8	468	R	416	r	252	i
9	468	R	416	r	333	R
11	468	R	3665	R	287	i
17	158987	r	252	i	B5	R
19	158987	r	287	i	B25	r
					B26	R
86	876	R	608	r	B656	r
					B657	R
88	876	R	808	R	B662	i
89	876	R	826	R	B655	r

The cells of all individuals in table 3, except from numbers 257, 333, B40 and B41 have been tested at least twice. The prefix B indicates that the individuals were born in 1953; other sheep are one year of age or more.

416 is *I/i r/r*, that of both 252 and 287 is *i/i R/r*. It is also apparent from the pedigree in figure 1 that ram Moore has the genotype *i/i R/r*, because matings with r individuals resulted in offspring of the R, r and i groups. Furthermore Nos. 332 and 333 must have the genotype *R/r* and the latter must be heterozygous *I/i*, because her lambs B23 and B24 from a mating to Moore were of group i. The origin of one of the genes for i in each of these twins can be traced back to one of their maternal grandparents. The other gene for i came from the sire (Moore). Similarly the *R* gene in B5, which was not expressed in its mother (252) because of the action of the homozygous *i/i*, was derived from its maternal grandfather. As shown in figure 2, the *R* gene in B26 can in a similar fashion be traced back to one of its maternal grandparents. When the ram of group i (Moore) was mated to two ewes of group i (as in figure 2), the offspring were of group i as expected. The breeding records of the flock showed that ewe 287 of figure 2 had been mated

to the ram Moore, and later to ram 158987. If the progeny No. 380 were sired by Moore, the gestation period was 150 days as compared with 168 days if 158987 were the sire. The gestation period in sheep is 140 to 160 days (DUKES 1947). Thus it seems somewhat more probable that the ram Moore is the sire of lamb 380, and this is indicated by the dotted line in figure 2 between

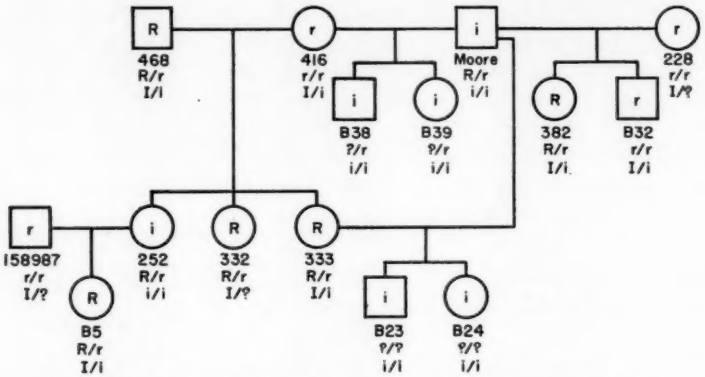

FIGURE 1.—Pedigree showing inheritance of R, r and i groups in sheep.

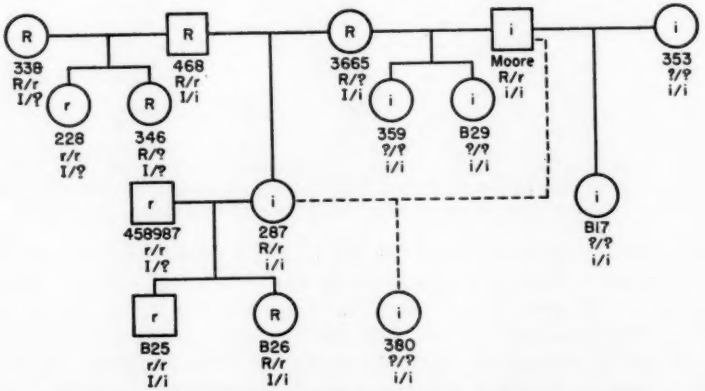

FIGURE 2.—Pedigree showing inheritance of R, r and i groups in sheep.

Moore and 287. The gene for i is undoubtedly not sex-linked, because ram 468 (*I/i R/r*) has two daughters (252 and 287) of group i.

The data of the present study are not well suited for studies of the gene frequencies, because they are derived from four small populations within each of which only a few rams have been used. However, it may be possible to get a rough estimate of the gene frequencies. Only ewes were used in the calculations because the lambs formed a small number of half-sib-groups. The

degree of relationship among the ewes has been disregarded. Each ewe has only been counted once, even if it has been used in more than one mating.

The numbers of ewes of the different breeds and their respective blood-groups are summarized in table 4. A χ^2-analysis was performed to test if the four subpopulations could be treated as one population in the calculations of the gene frequencies. The numbers of individuals of group i were very small. However, the ewes of group i in the Hampshire and Shropshire breeds have all been shown to be of the genotype *i/i, R/-*. Thus they were counted as R individuals. On the other hand the four ewes of group i in the Southdown and " Mixed " breeds have been omitted, because they could be any one of the genotypes *R/R, R/r* or *r/r*. The χ^2-test indicates that the data could be

TABLE 4

The blood groups of ewes of four different classes of sheep.

Breed	Number of ewes of group			Total number
	I		i	
	R	r		
Shropshire	15	10	2[1]	27
Hampshire	26	15	4[1]	45
Southdown	15	3	2	20
Mixed	37	16	2	55
Total	93	44	10	147

[1]These ewes have been proven to be of the genotype R/-, *i/i*.

pooled ($\chi^2 = 2.51$, D.F. = 3). The pooled gene frequencies were as follows: $R = 0.45$, $r = 0.55$, $I = 0.74$ and $i = 0.26$. The calculations have been made under the assumption that individuals of the genotype *i/i r/r* show the phenotype i. This question will be discussed below.

DISCUSSION

KACZKOWSKI (1928) and YCAS (1949) classified sheep bloods according to their reactions with the normally occurring anti-R. Thus they did not have the test fluid, or reagent, to distinguish between individuals of group r and i, neither of which react with anti-R. However, both authors subdivided these non-reacting animals (r and i in this paper) into two groups according to the presence or absence of anti-R in the serum. It might be expected that some individuals of group R would have arisen in the different matings between non-reactors reported by KACZKOWSKI and by YCAS. Actually, YCAS observed one individual of group R from such a mating. She suggested that the R lamb in this case was due to an error in the breeding records. However, such a result would be expected in the light of the present findings.

One possible explanation of the action of the gene for i might be that it prevents the secretion of the R and presumably the r-substance into the serum. This is somewhat analogous to the proposed action of the secretor gene (*S*)

in humans described by SHIFF and SASAKI (1932). Individuals which carry homozygous recessive gene (*ss*) do not have the A, B or O substances in their body fluids and secretions, such as saliva and tears, whereas these are found in all individuals carrying the normal allele (*S*), with the particular substance of the secretions dependent upon the genotype for A, B or O of the cells. Further, the antigens A, B, and O of the red blood cells of man are not known to be affected by the secretor (*S*) or nonsecretor (*s*) genes. In contrast, in the blood of sheep the presence of the R or r substances on the cells appears to be entirely dependent upon its prior appearance in the plasma. The genes for the R and r substances of sheep then, are capable of functioning, at least in the plasma and blood cells, only in the presence of the allele (*I*) to the recessive suppressor gene (*i*). By analogy with other genetic phenomena, it might be proposed that the gene *i* when homozygous in some way inhibits or suppresses an enzyme which is active in sheep with gene *I*. If this is correct, such an enzyme should be sought in an organ, or organs, as the liver, concerned with the synthesis of serum and serum constituents. Other explanations cannot be excluded, but this relatively simple one seems reasonable.

Eight of the 23 individuals of group i included in the present studies have been shown to be of the genotype *i/i R/-* because when mated to *r/r* they have produced offspring with R. The remaining 15 may be of any of the genotypes *R/R, R/r* or *r/r*. However, it cannot be established that any of these are of the genotype *i/i r/r*. Such an individual must result from a mating r × r. That no individuals of group i have occurred among the 44 offspring produced in the matings of r × r may be explained (a) by assuming that the three rams of group r are homozygous *I/I*, or (b) that the *i* gene in homozygous condition suppresses only the formation of the R-substance, not the r-substance. Only one of the three rams had sufficient offspring to conclude with reasonable certainty that it was *I/I*, while the other two could have been either *I/I* or *I/i*.

The R, r and i classes of blood in sheep have considerable resemblance to the Lewis blood groups in man, according to the explanation of the inheritance of the Lewis groups by ANDRESEN and JORDAL (1949). A recent proposal of the inheritance for the Lewis system has been advanced by GRUBB (1951) which, if applied to the R, r and i groups in sheep, would mean that the R and r substances are determined by a complicated interaction of genes at different loci, as R and R′ at one locus, r and r′ at another, the phenotype i being present if the genotype is *R′/R′ r′/r′*. The most pertinent argument against such an explanation for sheep bloods is the appearance of R, r and i offspring from r × i matings, as was actually observed. For this and other reasons, it seems much more reasonable to explain the situation by assuming that the R and r substances are produced by alleles, and that a recessive gene *i*, at another locus, when homozygous suppresses the expression of the R substance, and presumably also the r substance.

Another interesting aspect of the R, r and i groups of sheep blood is the observation that all sera from individuals of group r have contained R-anti-

bodies, while only two out of 23 individuals of group i showed anti-R. BERNSTEIN (cited by WIENER 1943) postulated that the antithetical relationship in humans between α-agglutinin and antigen A, and between β-agglutinin and antigen B, could arise under the assumption that all humans produced α- and β-agglutinin, but that α-agglutinin is absorbed if antigen A is present on the cells, as is β if the cells possess β. If such a system were operating in sheep to determine the presence of R-antibodies, one would expect all sheep of the groups r and i to contain anti-R. Since only two of the twenty-three individuals of group i possessed anti-R, it appears highly probable that the explanation proposed for the normal antibodies of humans is not valid for sheep. Further, there remains the possibility that the gene for the r substance has a pleiotropic effect, to produce the r substance in the plasma in the presence of the *I* gene, and anti-R in the serum. Under this proposal, which parallels a similar explanation for the reciprocal appearance of the iso-agglutinins and the A and B antigens of humans (COTTERMAN 1952, among others) group i animals of the genotype *i/i R/-* should not, while those of the genotype *i/i r/r* should, contain anti-R in their sera. This possibility can be substantiated or disproved only after the sera of sheep of the genotype *i/i r/r* can be tested.

On the assumption that the explanation proposed above of the inheritance of the R, r and i classes of the bloods of sheep is correct, it provides an example of a recessive gene (*i*), epistatic to genes (*R* and *r*) which effect antigenic substances of the plasma and secondarily of the blood cells. If the epistatic action of the *i* gene had its primary action on the cells, it would represent a different effect from any known at present of genes which produce cellular antigens. In the light of the present evidence, the authors favor the explanation that the *I* and *i* genes act on the secretion of R and r substances into the serum, from which they are taken up by the blood cells.

SUMMARY

The present report describes three classes of the blood of sheep. (1) R blood, characterized by the R-substance on the cells and the soluble R substance in the serum. The R factor is detected by normally occurring antibodies in some sheep lacking R. (2) r blood, having the r-substance on the cells and the soluble r substance and R-antibodies of varying strength in the serum. Some cattle normal sera contain specific antibodies against the r-factor. (3) i blood, having neither R nor r substances in the serum or on the cells. Only some individuals of this group have R-antibodies in their sera.

The R and r substances are not detectable on the cells of lambs until they reach an age of about one month. The respective substances in soluble form are present in the sera before they can be detected on the cells.

It has been possible *in vitro* to change the cells of one group into a second group, by incubating them in serum of the second group. It has thus been possible to produce an artificial cell-type reacting with both anti-R and anti-r sera, by incubating r cells in R serum. These experiments and the gradual development of the R and r substance on the cells of lambs indicate strongly

that the R and r substances are taken up by the cells from the surrounding plasma.

The data on the inheritance of the three classes of blood, R, r and i, are in accord with the assumption that it is controlled by genes at two loci, with the recessive gene *i* epistatic to dominant *R* and probably also to *r*, so that group R is *I/- R/-*, r is *I/- r/r*, and i is *i/i R/-* (and probably *i/i r/r*).

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MUTATION RATES AT SPECIFIC AUTOSOMAL LOCI IN THE MATURE AND IMMATURE GERM CELLS OF DROSOPHILA MELANOGASTER[1]

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STUDIES utilizing mutation rates at specific loci as a measure of radiation damage to biological material allow a systematic test of limited sections of genetic material. The damage is measured in terms of gene mutations, since these could be retained in the gene pool of a population and possibly be expressed over a number of generations.

A differential sensitivity to X-radiation for the different stages of spermatogenesis of Drosophila has been suggested by a drop in the number of sex-linked lethals obtained from mature males 14 to 16 days after treatment (HARRIS 1929; HANSEN and HEYS 1929). MOORE (1934) found fewer sex-linked visible mutations from adults which had been treated as larvae than from treated adults. The present study was designed to determine mutation rates at 8 autosomal loci in sperm and spermatogonial cells of *Drosophila melanogaster*. The early larval stages were treated to limit the test to spermatogonial cells and the mature sperm test was limited by using the males for only 4 days after treatment. These procedures, including the use of specific loci, were employed to avoid some of the complications encountered in previous studies of this type.

The specific loci test can be used to provide a reliable index for comparisons of genetic damage produced by X-radiation with that produced by other types of radiation. It is also the best of the few general methods for comparative studies of radiation damage in different biological forms.

MATERIALS AND METHODS

General procedure

A standard stock of *Drosophila melanogaster*, designated Oregon-R, Oak Ridge, was established from the Oregon-R stock by inbreeding pairs for 4 generations, then combining several of the most fertile strains from each of the original pairs. The mutant test stock was the third chromosome *res* complex. The linkage positions (BRIDGES and BREHME 1944) are as follows: *ru* (roughoid), 0.0; *h* (hairy), 26.5; *th* (thread), 43.2; *st* (scarlet), 44.0; p^p

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(peach), 48.0; *cu* (curled), 50.0; *sr* (stripe), 62.0; e^s (sooty), 70.7. Mutations of spontaneous origin were chosen in order to reduce the probability that deletions or chromosome aberrations were associated with the mutants. A cytological check of the Oregon-R and *res* marker stocks by C. L. WARD showed that there were no inversions or detectable deletions.

The general procedure for establishing mutation rates consisted in mating X-irradiated males to homozygous *ru h th st* p^p *cu sr* e^s females. Any mutation at any one of the 8 loci as well as dominant mimics and modifiers, phenocopies, and partials can be detected in the F_1 individuals.

The procedure for testing the F_1 variants for classification into one of several categories is as follows:

P_1 Oregon-R ♂ treated × *ru h th st* p^p *cu sr* e^s (*res*) ♀

1. Test of F_1 ♂ variants:

   F_1 ♂ variants (*h′* induced) × *res* ♀♀

   F_2 classes:

   Presence of *h′* or *h* in all offspring excludes dominant mimics and modifiers on the X, second and fourth chromosomes, phenocopies and partials excluded.

   Crossover test: (pairs tested)

   F_2 (*h′/res*) ♀ × *res* ♂

   F_3 classes:

   Presence of *h′* or *h* in all offspring repeats previous test and excludes dominant mimics and modifiers on the third chromosome except any which may be so close to the *h* locus that no crossovers are detected. Crossover values may not be normal because of the presence of induced inversions or translocations. Normal individuals indicate a dominant mimic or modifier on the third chromosome.

2. Test of F_1 ♀ variants:

   F_1 ♀ variant (*h′* induced) × F_1 ♂♂ (treated chromosome/*res*)

   F_2 classes:

   Normal individuals received treated chromosome from F_1 ♂.

   Crossover test—detectable by presence of *h′* or *h* alone or with any one or combination of *res* mutants:

   This test excludes all nonmutant variants as regular crossover test. Presence of any one or combination of *res* mutants without *h′* or *h* indicates a nonmutant type.

   Additional crossover test:

   Strains of the original mutant were obtained by crossing individual F_2 ♂ (*h′* or *h/res*) to *res* ♀♀. Individual ♀ *h′* or *h/res* (from F_2 ♂ strains) × *res* ♂.

   Classes obtained:

   Presence of *h′* of *h* in all offspring excludes all nonmutant variants as in other crossover test.

The crossing-over data from the F_1 female variant gave immediate proof as to whether or not the variants were true mutations but the crossing over introduced difficulties in establishing stocks of the induced mutations, since the marker mutant may have crossed into the irradiated chromosome. Individual F_2 males which showed the mutation (either induced or from the *res* complex) were crossed to *res* females, a minimum of 10 strains from individual F_2 males being established whenever possible. The greater the number of mutant strains established, the smaller the chance that all of them would contain the allele from the *res* complex. The center mutants of the *res* complex, *th, st, p^p, cu* and *sr* would require a double crossover to be crossed into the treated chromosome, but only a single exchange would be necessary to recover *ru* or e^s alone. Since the region between *sr* and e^s is 8.2 units long about 8 individuals in a sample of 100 will show a single or multiple exchange involving this region. The *ru* mutant from *res* would be more likely to be crossed into the treated chromosome, since the *ru-h* region is 26.5 units in length. The induced allele could be distinguished phenotypically or cytologically from the tester allele in some cases, thus insuring its recovery. In only 1 or 2 cases the induced mutant resembled the test mutant so closely that the two could not be distinguished phenotypically or cytologically. A viability test eliminated a large number of doubtful cases. A lethal, semilethal, or sterile condition of the homozygous mutant showed that the induced mutant was recovered. Up to 9 strains of certain mutants were tested and all strains of a given mutant gave the same viability result. It should be noted that the possibility of crossing out the *res* mutants does not affect the determination of the mutation rate since the crossover tests prove the original variant to be a true mutation. Crossing over affects the possible validity of the viability test only.

Spermatogonial test

Mature sperm as well as three types of immature germ cells, spermatids, spermatocytes, and spermatogonia are present in the adult males of Drosophila. In irradiated adult males, there is no sure way to tell which type of immature cells is being tested. To ensure that spermatogonia were tested, larvae which contained only spermatogonia were irradiated. According to KERKIS (1933) and GLOOR (1943), only spermatogonia are present in the larval gonads 24 hours after the larvae hatch from the egg at a temperature of 25°C, the first spermatocytes appearing about 48 hours after the larvae hatch from the egg.

Aged Oregon-R, Oak Ridge flies (usually 4 to 7 days old) which had produced larvae in culture bottles were placed in empty pint milk bottles. These bottles were then inverted in Petri plates lined with damp filter paper which was used as a source of moisture. A piece of damp filter paper the size of the mouth of the bottle and coated with a mixture of yeast-banana agar, Y-2 yeast, and a drop of acetic acid added to stimulate egg laying, was used for collecting eggs. The flies were allowed to lay for a 2-hour period, after which

the filter papers with the eggs were removed to small culture vials containing banana-agar food. To provide moisture necessary for normal hatching a small amount of saline was placed in the vial with one edge of the filter paper touching the water. After 21 hours at 24°C, the filter paper was removed and any larvae which had hatched in this period were washed off with saline and discarded. The remaining eggs were then allowed to hatch over a 2-hour period and these larvae were transferred onto a large piece of filter paper which contained a mushy solution of banana-agar food and yeast. In order to ensure that all larvae would be of uniform age, great care was taken to allow no unhatched eggs to remain. The larvae were aged for 20 hours in a glass Petri plate and transferred to a lucite Petri plate for X-irradiation. These 20- to 22-hour larvae were irradiated with 900 roentgens of 250 kvp X-rays delivered by a Coolidge self-rectifying tube with inherent filtration of 3 mm of aluminum and a half value of 0.4 mm of copper. Using a current of 15 ma, the intensity obtained at 84 cm distance was approximately 85/r/minute.

A dose of 900 r was decided upon after a preliminary test of the viability and fertility of adults which had been treated as larvae. Doses of 800 and 900 r gave 60 to 75 percent recovery of adults from treated larvae. Only a few larvae died in the pupal stage and the fertility of the adult males was approximately 80 percent.

Increasing the dose to 1000 r caused a tremendous increase in the death rate of pupae and a drop to about 40 percent in the fertility of the males. Raising the dose to 1200 r caused no further decrease in the number of adults that hatched but these adults were very weak and the fraction of fertile males dropped to about 20 percent.

After X-irradiation with 900 r the larvae were placed in a bottle containing yeast-banana-agar food. The adult males, which hatched from these larvae were aged 6 to 7 days and were mated individually to several *res* females in all experiments but one. In this case each male was mated successively, to a single different female for a 24-hour period until four such matings were made.

Information on the effect of irradiation on spermatogonia was obtained from 7 experiments in which from 126 to 400 P_1 males were tested. In the first larval test, in which there was no way to determine the number of mutations to expect from the sample, an average of 290 F_1 offspring from each male were checked. After recovery of several cluster mutations the sample size was reduced to 113 to 140. Offspring from each male were recorded separately. Males from which one or more mutations were recovered were retained and larger samples obtained for determination of cluster sizes as expressed in the ratio of mutant to total offspring. Several mutant stocks were established from each cluster, using F_1 male mutants when possible. These mutants were tested as described under *General procedure*. The mathematical treatment of spermatogonial mutation rates will be discussed under Mutation rates.

Sperm test and control

Adult males, 6 to 7 days old, were X-irradiated with 3000 r and mated, in pairs, to *res* females. After 24 to 36 hours the males and females were separated and each male mated to another *res* female. Each treated male, remated every 24 hours, was mated in sequence to a total of four *res* females over a period of 96 hours and then discarded. This technique was employed to detect any spontaneous mutations which might be carried heterozygous by the male and to limit testing to the later stages of spermatogenesis—presumably, only mature sperm. The F_1 offspring of each male were recorded separately. Variants which resembled any of the 8 mutants were tested as described under General procedure.

Such a paired-mating technique is not sufficient to detect spontaneous clusters in the treated males, as evidenced by the recovery of 2 different mutations at the same locus from the progeny of a single treated male and of recovery of mutations at 2 and 3 different loci from the progeny of a single male. The separate control test must therefore serve as a comparison test. Even though spontaneous clusters cannot be detected by this type of test, it does serve to limit the sperm sample obtained from the male. Larvae for the control, mature sperm and spermatogonial tests were collected in the same way to prevent any possible physiological difference in the 3 tests. Adult males for the control test were mated in the same way as those in the mature sperm test.

Viability test

X-ray-induced mutations recovered from spermatogonia and mature sperm were tested for viability in the homozygous condition. Only 1 strain of the mutant was tested if the mutant originated from a male; 1 to 9 strains were tested if the mutant was obtained from a female. In most cases the spermatogonial clusters produced a few mutant males so that stocks of these could be established from males.

An ebony mutant was used to test all induced mutants except that peach was used for those at the ebony locus. The mutant strains which had been carried through the male heterozygous with *res* were crossed to ebony-11 or peach. Ten to 25 paired matings of the F_1 phenotypically normal individuals were used. Genetically, the F_1's were heterozygous for the test mutant, ebony or peach, and the mutant being tested. A mutant ratio of 1 test mutant: 2 normal: 1 induced mutant would be expected from such a cross if the induced mutant survived in a homozygous condition and was as viable as the test mutant. A ratio of 1 test mutant: 2 normal with the third class absent showed that the induced mutant was homozygous lethal. A large reduction in size of the induced mutant class indicated lowered viability; this condition was considered semilethal. Ratios of 1 test mutant: 3 normals indicated that the mutant was not phenotypically detectable in a homozygous condition, even though the mutant phenotype was expressed when heterozygous with the mutant contained in the *res* complex. This method has an advantage over a balancer

method since crossing over would decrease the chance of lethals, present at another locus, being homozygous in all offspring.

A different method was necessary to test the *sr* mutants, since some ebony allele must be present in a heterozygous or homozygous condition for the mutant to be detected phenotypically. For testing the *sr* mutants the *sr/res* males were mated to $Dl^{13}/In\ (3R)C,\ Sb\ e\ l(3)\ e$ females. The phenotypic *Sb*, non-ebony F_1 individuals were mated in pairs. The appearance of normal non-*Sb* F_2 individuals showed that the homozygous *sr* mutant lived since the balancer chromosome contained a lethal and cannot be recovered in a homozygous condition. The heterozygotes of the mutant and balancer showed *Sb*. The normal F_2's (homozygous *sr*) were crossed to *res* to test whether the mutant was present homozygous or crossing over had occurred to give normal individuals.

RESULTS

The F_1 variants recovered from the mature sperm test are included in table 1. The variant was placed in the " Mutations " category if it could be proved by the crossover test to involve the locus which it phenotypically re-

TABLE 1

Variants recovered from mature sperm X-irradiated with 3000 r (Sample size: Control, 45,504; mature sperm, 49,512).

Variants	Loci tested								Total
	ru	*h*	*th*	*st*	p^p	*cu*	*sr*	e^s	
(Control)	0	0	0	0	0	0	0	0	0
Genetic mutations	3	6	4	9	13	8	12	13	68
	(2)			(1)					(3)
Phenocopies and mimics	89	1	2	67	3	10	8	17	197
Sterile variants	35	5	0	10	9	3	7	7	76
Dead before completion of test	65	5	3	9	4	10	14	4	114
Partials	19	1	0	0	2	3	7	0	32
Total	213	18	9	96	31	34	48	41	490

sembled. This type of variant was the only one used for calculating mutation rates. A total of 71 mutations was recovered from a sample of 49,512 F_1 offspring. Of 324 males tested, 262 produced no mutation, 54 produced 1, 7 produced 2, and 1 produced 3 different mutations. The 3 mutations in parentheses were lost before a stock strain could be established but the number of crossovers checked was considered sufficient to prove them mutations.

Many variants were semisterile and produced only a small number of offspring. Other variants, tested for 2 or 3 weeks and remated several times to the *res* stock, proved to be sterile. Both of these classes were placed in the " Sterile Variants " category. Variants which died in 2 weeks of testing were recorded in the " Dead " column. The " Phenocopies and Mimics " category includes those variants which were not inherited but were due to physiological conditions and those which involved some other locus or loci in the genome.

The " Partials " included variants with unilateral and partial expressions which were not inherited.

According to this classification of variants the total number of mutant and sterile variants happens to be about equal but the same relation does not exist for each of the 8 loci. The roughoid phenotype accounts for about one-half the total variation in each of the nonmutant classifications but is next to the smallest class of mutations. A rough eye is obviously an exceptionally frequent phenocopy resulting from irradiating Oregon-R, Oak Ridge stock. This was not true for other phenocopies of mutants except scarlet. This complete lack of correlation between the numbers of mutant and nonmutant variants for the different loci requires genetic testing of all variants. Also, it is impossible to assign with assurance the sterility effects to the loci which are seemingly involved or to decide how many of the sterile variants might be true mutations.

TABLE 2

Variants recovered from mature sperm X-irradiated with 3975 r (Patterson, 1934, unpublished). (Sample size, 50,159).

Variants	Loci tested								Total
	ru	*h*	*th*	*st*	*cu*	*sr*	e^s	*ca*	
Genetic mutations	16	1	3	8	3	4	13	22	70
Phenocopies and mimics	7	0	0	1	6	1	1	7	23
Sterile variants	17	1	10	5	4	8	8	8	61
Dead before completion of test	1	0	1	1	1	0	1	0	5
Partials	3	1	2	1	2	2	1	4	16
Total	44	3	16	16	16	15	24	41	175

The absence of any noticeable drop in the number of mutations recovered —a drop would be an indication of the exhaustion of sperm which were mature at the time of treatment—indicates that only mature sperm (or equally sensitive late stages) were recovered within the 4-day period after irradiation of the adult male. The total number of 71 mutants were distributed over the 4 days of testing to give values of 0.149 percent for the first day, 0.111 percent for the second, 0.148 percent for the third, and 0.172 percent for the fourth day.

Table 2 includes unpublished data which were obtained by J. T. PATTERSON of the University of Texas in 1934. PROFESSOR PATTERSON has consented to allow these data to be included since 7 of the 8 loci used in his experiments were used in the present study. This permits a comparison of the mutation rates in mature sperm at these 7 loci in the Oregon-R, Oak Ridge and in a Texas strain of *D. melanogaster*. The *ru, h, th, st, cu, sr* and e^s loci were used in both experiments. The mutant, *ca* (claret) is located to the right of the e^s locus, at 100.7 on the third chromosome. A dose of 3975 r of X-radiation was used in the PATTERSON experiment and a sample of 50,159 individuals was checked.

Tables 1 and 2 show that the phenocopy and mimic variants were only about one-third as frequent as the mutations in PATTERSON's experiment using the Texas strain as compared with experiments using the Oregon-R strain. The sterile and mutation categories were fairly similar in the 2 experiments. The range and frequency of the nonmutant types of variants differed in the 2 normal stocks tested; however, this difference is unimportant in comparing mutation rates.

In table 3 the 95 percent confidence intervals for the frequency of mutation at the various loci are compared. These values were obtained from MOLINA's tables (Poisson's Exponential Bionomial Limit—MOLINA 1942) and STEVENS' tables (FISHER and YATES 1949). Data for the Oregon-R stock indicate possible but not certain differences in mutation rates among some of the loci. An overlap is found between the lower limits of the value for the loci with the highest observed frequency (e^s and p^p) and the values for other loci. The

TABLE 3

Comparative frequencies of mutations at specific loci.

Locus	Frequency			95% confidence limits		
	Observed		Adjusted			
	Oregon-R (3000 r)	Texas (3975 r)	Texas (3000 r)	Oregon-R (3000 r)	Texas (3975 r)	Texas (3000 r)
ru	5	16	12.08	1.62–11.67	9.14 –25.99	6.90–19.62
h	6	1	0.75	2.20–13.06	0.025– 5.57	0.02– 4.20
th	4	3	2.26	1.09–10.24	0.62 – 8.77	0.47– 6.62
st	10	8	6.04	4.80–18.44	3.45 –15.81	2.60–11.93
p^p	13	....	....	6.92–22.27	....	....
cu	8	3	2.26	3.45–15.81	0.62 – 8.77	0.47– 6.62
sr	12	4	3.02	6.20–20.97	1.09 –10.24	0.82– 7.73
e^s	13	13	9.81	6.92–22.27	6.92 –22.27	5.22–16.81
ca	....	22	16.60	....	13.79 –33.35	10.41–25.17

data for the Texas strain show greater differences among the mutation frequencies of some of the 8 loci. The 8 mutants can be divided into the following general classes, on the basis of frequency: *ca* and *ru* loci have high frequencies; e^s and *st* loci have somewhat lower frequencies; mutations at the *h, th, cu,* and *sr* loci occurred still less often. The 4 loci with the lowest frequencies have about the same range, and the confidence intervals do not overlap that of *ca*. The other loci are intermediate in that the confidence intervals overlap either the high *ca* range or lower values of *h, th, cu* and *sr*.

A comparison of mutant frequencies between the Oregon-R and Texas strains (mutation rate for Texas strain has been adjusted for 3000 r) shows that the confidence intervals for similar loci overlap in all 7 cases. The greatest differences exist between the mutation rates of p^p and e^s in the Oregon-R strain and among those of *h, th, cu* and *sr* in the Texas strain. The 95 percent confidence interval for the total number of mutations at the 7 similar loci in the 2 experiments overlap. In this comparison the p^p locus in the present experiment and *ca* locus in PATTERSON's experiment were omitted. After omission

of 13 p^p mutants the 95 percent confidence interval for the remaining 58 is 44 to 75. In PATTERSON's experiment, 48 mutations were found, excluding the 22 at the *ca* locus. Adjusted to the radiation dose used in the present investigation, a rate of 36.2 mutations with 27 and 48 as the 95 percent confidence limits was obtained.

Data for spermatogonia presented in table 4 include 10 separate mutations recovered from 1797 males which were irradiated as larvae. In 3 cases a single mutant individual was recovered from the total sperm sample; in the remaining 7 cases 2 or more mutants were detected. The actual number of mutants recovered from the total sperm sample (total number of offspring recovered from the male) in each case is given in the third column. From the ratio of mutant to normal in the total sperm sample, given in the fifth column of the

TABLE 4

Spermatogonial mutations.

Locus	Number of independent occurrences of mutation	Number of mutant individuals in each sample	Total number of individuals in each sample	Ratio of mutant in total sperm sample
b	2	5	598	1/120
		17	770	1/45
cu	2	1	225	1/225
		4	297	1/74
sr	3	2	161	1/80
		1	342	1/342
		2	181	1/90
e^s	3	31	469	1/15
		1	110	1/110
		18	388	1/21

Total number of males tested = 1797.
Total number of sperm tested = 273,574.

table, the number of spermatogonia present in the adult male can be estimated. Each spermatogonium contains 2 sets of chromosomes; therefore the number of spermatogonia is obtained by dividing the denominators of these ratios by 2. The 1/120 ratio obtained in the first hairy cluster indicates that 60 spermatogonia were present in the males; the other hairy cluster gives a value of 23 or 24 spermatogonia. The wide variation in the ratios of different clusters shows that the number of spermatogonia also vary. Judged by this type of estimation the number of spermatogonia vary from a low of 7 or 8 to more than 100 per male.

Viability test

Table 5 contains the data for the viability test of the X-ray-induced mutations. Twenty of the 58 tested mutations induced in mature sperm lived in a homozygous condition, 35 were lethal, and 3 were semilethal. The ratio of viable to lethals would therefore be 1 : 1.8. About one-half of the mutations

TABLE 5

Viability of homozygotes of mutations obtained from X-irradiated sperm and spermatogonia.

	Loci tested								Total
	ru	*h*	*th*	*st*	p^p	*cu*	*sr*	e^s	
Sperm									
Viable	1	1	0	1	5	5	0	7	20
Lethal	3	2(1)	4	7	3	1	7(1)	5(1)	32(3)
Semilethal	0	1	0	0	1	1	0	0	3
Total	4	4(1)	4	8	9	7	7(1)	12(1)	55(3)
Spermatogonia									
Viable	....	0	....	....	....	2	0	2	4
Lethal	....	1	....	....	....	0	2	0	3
Semilethal	....	1	....	....	....	0	0	0	1
Total	....	2	....	....	....	2	2	2	8

from spermatogonial tests were viable in a homozygous condition and one-half lethal or semilethal. The finding of the 3 conditions, lethal, semilethal, and viable among spermatogonial mutants is important although the numbers are not large enough to determine their true proportions.

Mutation rates in mature sperm

The mutation rates (table 6) are expressed as the rate of mutation per roentgen of X-radiation of each locus. The rate for mature sperm was obtained by dividing the number of mutations produced at one locus by the number of individuals checked and the total dose in roentgens. The average rate for all 8 loci tested was obtained by dividing the total number of mutations recovered by the dose, number of individuals checked, and the number of loci used. An average mutation rate for these third chromosome genes of 5.72×10^{-8}/r/locus is obtained if the 3 cases are excluded in which stocks were not established. The rate is 5.98×10^{-8}/r/locus when they are included.

The mutation rates at specific loci varied from 2.01×10^{-8}/r to 8.75×10^{-8}/r. The variation between the different loci may be significantly dif-

TABLE 6

Mutation rates at specific loci and average rate for the third chromosome of Drosophila melanogaster.

Sample size and dose	Rate at specific loci/r $\times 10^{-8}$									Average rate/r $\times 10^{-8}$
	ru	*h*	*th*	*st*	p^p	*cu*	*sr*	e^s	*ca*	
Oregon-R strain										
49,521	2.01	4.04	2.7	6.06	8.75	5.4	8.08	8.75	....	5.72
	(3.4)			(6.7)						(5.98)
3,000 r										
Texas strain*										
50,159	8.02	0.5	1.5	4.01	....	1.5	2.0	6.5	11.03	4.4
3,975 r										

* Patterson, unpublished.

ferent from chance variation but no exact statistical test is available. There was no correlation between the mutation rate and the cytological position of the gene on the chromosome.

A similar treatment of PATTERSON's data gives an average rate of 4.39×10^{-8}/r/locus (table 6). A statistical comparison of the two experiments has been considered previously.

Mutation rates in spermatogonia

Several characteristics of Drosophila spermatogenesis must be discussed before the mathematical treatment of mutation rates for spermatogonial cells can be considered. In the spermatogenesis of *D. melanogaster* the primary germ cells migrate from the polar cap in the developing egg to the testes of the early larvae. SONNENBLICK (1941) states that a number of the primordia are lost in this migration. RABINOWITZ (1941) gives an average number of 40 primordia which begin this migration. The number of cells which are incorporated into the gonads fall into 2 groups, for some gonads contain 5 to 7 cells, others contain 9 to 13 germ cells. Between the formation of the testes and hatching of the egg 1 or 2 mitoses occur. The larvae at the time of hatching from the egg may have gonads with 8, 10 or 12 primordia while others have 36 to 38 primordia per gonad (SONNENBLICK 1941). The histological data thus show that the number of primordia incorporated into these early larval gonads varies, or that a different number of divisions has occurred. KERKIS (1933) and GLOOR (1943) report that only spermatogonia are present 24 hours after the larvae hatch from the egg. KERKIS's observation of the presence of many division figures at this stage indicates a rapid multiplication of the spermatogonia. Granted that mitotic divisions occur as indicated by KERKIS's data, it is still not known whether each spermatogonial division occurs at the same time or whether there is an asynchronous type of division. Each spermatogonium could divide the same number of times before producing spermatocytes, or some spermatogonia could undergo more divisions than others. It should be noted that the larvae were X-irradiated during this early stage of spermatogenesis, that is, 20 to 22 hours after hatching from the egg.

The first spermatocytes are produced from the spermatogonia about 48 hours after the larvae hatch from the egg. Primary spermatocytes are produced in groups of 16 from 4 synchronous divisions of each spermatogonial element; from these groups, 64 haploid sperm are produced (PONTECORVO 1944; TIHEN 1946). Since there is no crossing over in the male, half the sperm (32), contain similar haploid strands from one chromosome and half contain similar strands from the other, except when a spontaneous mutation had occurred. Therefore, when a mutation is produced in the spermatogonial stage, a number of sperm with the same mutation may be recovered in the mature sperm sample. The spermatogonia continue to form spermatocytes which in turn form mature sperm. Any mutation present in the spermatogonia would also continue to appear in the sperm as long as the spermatogonial stem cell is active.

Genetic data on the cluster mutations offer the only method of studying the type of sampling obtained from the spermatogonia and also the relative activity of the mutant and nonmutant spermatogonia. Only 2 of the cluster mutations were suitable for such study. The affected male with an *ebony* cluster was mated to several *res* females and remated several times to new females. Offspring were collected over a period of a month. The following mutant and normal offspring were collected at 4 successive remating intervals.

Number of mutants	*Total sample*	*Ratio*
7	146	1:21
5	142	1:28
14	126	1:9
5	55	1:11
31	469	1:15

In the second cluster, involving the mutant hairy, the affected male was remated to a separate female every 24 to 36 hours; the ratios are based on the total number of mutant sperm recovered among offspring from each female. The time can be expressed in terms of successive (daily) fertilizations by the male.

Female No.	*Mutants*	*Total sample*	*Ratio*
1	2	108	1:54
2	6	239	1:40
3	2	202	1:101
4	3	88	1:29
5	4	133	1:33
	17	770	1:45

Homogeneity within the 2 clusters was measured by computing the χ^2's. For the first cluster, $\chi^2 = 7.66$ which for three degrees of freedom corresponds to a probability of .056. In the second cluster, $\chi^2 = 2.53$ which for 4 degrees of freedom corresponds to a probability of .63. Since the smallest expected frequencies in the first and second clusters are 3.63 and 1.94, respectively, the χ^2 approximations may be inaccurate. As a rough guide, however, homogeneity is indicated in the second cluster; the value for the first cluster is in the questionable range.

The genetic data from these two clusters show a rather random distribution of mutant sperm among the normal sperm. No large clumps of mutant sperm were recovered in any one part of the sample. Sperm produced from a number of different spermatogonia must be mixed in the vagina of the female when insemination occurs or large clumps of mutant sperm (32 are produced from one division of the mutant spermatogonia) would have been detected. LÜNING (1952b) has also reported a mixing of sperm from different inseminations. This mixing of sperm (from different stem cells) in the vagina obscures testing to determine the synchronization of the stem cell divisions. However, synchronous and asynchronous types of divisions can be inferred from the consistency or variation in the ratios of mutant and normal sperm over the sampling period. Asynchronous divisions of a few nonmutant spermatogonia,

which are in a relatively larger number than the mutant spermatogonia, would produce slight variations in the ratio of mutant to normal sperm and would be more difficult to detect than an additional division of the 1 mutant spermatogonium. The stem cell divisions may not be perfectly synchronized but, whatever the mechanism or mechanisms may be, the ratios vary no more than might be expected from chance. The absence of any progressive decrease in the number of mutant sperm as compared to normal sperm throughout the sampling period shows that mutant spermatogonium is not becoming inactive before nonmutant spermatogonia. These two clusters showed no selective action against the mutant spermatogonium in this respect.

Estimation of the mutation rate for spermatogonia is complicated by the following fact: The sample of F_1 flies collected from each male, which had been treated when a larva, was usually between 115 and 150. However, if a mutant individual was detected in this sample, the male parent was retained and remated. Most of the samples of F_1 progeny that contain mutants (column 4, table 4) are, therefore, considerably larger than those which contain no mutants. The most satisfactory method for estimating the mutation rate seems to be to calculate the mean of the rates found in the progeny of each of the 1797 males. Since most of the males had no mutant offspring, this mean is simply

$$\frac{1}{1797}\left(\frac{5}{598}+\frac{17}{770}+\ldots+\frac{18}{388}\right)=1.09\times10^{-4},$$

for all 8 loci and for 900 r, or 1.52×10^{-8}/r/locus. The expression within brackets is obtained from columns 3 and 4, table 4. This estimate is, however, biased. If the samples in which mutants were found had not been augmented, then cases in which a mutation occurred in the spermatogonia but was not present in the sperm sampled, would have been compensated for by a corresponding excess proportion of mutants in the samples that contained mutants. With augmented samples this compensation will not be complete and the above method of calculating the rate will be expected to underestimate it. However, in the present data, this bias is not likely to be large, and probably does not invalidate the conclusions drawn from the estimated rate.

To facilitate the estimation of confidence limits, the value for the rate may be regarded as approximately what would have been obtained if the calculation could have been made from 10 observed occurrences of mutation in the (actually unknown) number of chromosomes tested. The 95 percent confidence limits of 10 are 4.80 and 18.39 (FISHER and YATES 1949). Expressing these in terms of the rate of 1.52×10^{-8}, they become $(0.728 \text{ and } 2.79)\times10^{-8}$. Since the upper limit falls well below the lower limit of the 95 percent confidence interval for the mutation rate in mature sperm, it appears that, even when allowance is made for the bias in the estimate of the spermatogonia rate and for error in the estimation of its confidence interval, the rates in sperm and spermatogonia are significantly different.

DISCUSSION

The previous studies on mutation rates at specific loci in Drosophila have used mature sperm and have often utilized loci with high mutation rates. The highest rate reported for any locus of *D. melanogaster* is that of the white locus by TIMOFÉEFF-RESSOVSKY (1933). A rate of 15 to 20×10^{-8}/r was obtained for this locus when the allele from one strain, designated as American strain, was tested. A mutation rate of 11×10^{-8}/r was obtained for the same locus when another strain was tested. BONNIER and LÜNING (1949) obtained a rate of approximately 8×10^{-8}/r for white if only those mutants which had been tested by progeny tests were included. The rate approached 12×10^{-8}/r when the untested variants were included. The same approximate values were obtained for the forked locus in the same experiment. TIMOFÉEFF-RESSOVSKY and DELBRÜCK (1936) obtained a rate about one-half this value for forked but VALENCIA and MULLER (1949) obtained a value around 2.5×10^{-8}/r for the same locus. These studies show that different alleles of the same gene may have different mutation rates and may produce different patterns of mutant alleles (TIMOFÉEFF-RESSOVSKY 1933). The high mutation rates obtained for these genes seem to represent the upper range of Drosophila mutation rates. Three loci, pink, curled, and ebony, of the 8 tested in the present study gave rates of around 8×10^{-8}/r but the remaining 5 gave lower rates. The roughoid and claret loci of PATTERSON's study gave high rates but the hairy locus gave a rate of 0.5×10^{-8}/r. Rates lower than that of the hairy locus were obtained for the sex-linked mutants, *ras*, *sc* and *ac* by VALENCIA and MULLER (1949). An average rate for a number of loci, some of which have high rates and others which have low rates, will give a better general estimate for mutation rate in Drosophila. The average rates of 5.98×10^{-8} and 4.39×10^{-8}/r found in the present experiment and by PATTERSON therefore seem more representative than rates at a specific locus.

A differential sensitivity of mature and immature germ cells of Drosophila to X-ray treatment has been indicated by the lower number of mutations recovered from immature than from mature cells. A drop in the number of sex-linked lethals recovered from irradiated adults has been found by several workers (HARRIS 1929; HANSEN and HEYS 1929; DEMEREC and KAUFMANN 1941) to occur 14 to 16 days after treatment in *D. melanogaster*. This drop was explained by HARRIS (1929) as either germinal selection against the lethal or differential sensitivity of the two stages to X-radiation while HANSEN and HEYS believed the difference to be due solely to the latter explanation. KOSSIKOV (1937) found that the same type of drop in the number of sex-linked lethals occurred 6 days after treatment when adult males of *D. simulans* were irradiated. He attributes a differential sensitivity to the mature and immature cells and bases the possibility of selection against hemizygous lethals in the immature cells on the comparison of data from irradiated males and females. Since the reduction division does not occur in eggs until after fertilization, his data from females are not entirely comparable to those for the male.

The work of SHAPIRO and SEREBROVSKAIA (1934) and SHAPIRO (1936) on induced lethals of the X and second chromosome in mature and immature germ cells offers data which might indicate that both factors may be involved to produce the differences found between the X chromosome and autosomes. Adult males of *D. melanogaster* were irradiated and mated to appropriate marker females. The data from the first 6 days of the test were designated for the mature sperm test. New females were crossed to the treated males after 6 days and were left with the males for 14 days. The males were remated to virgin females on the twentieth day and data were collected from the twentieth- to twenty-sixth-day mating periods for immature cells. The comparative frequencies of recessive lethals in the X and second chromosomes were found to be 1 : 2.66 for the mature sperm and 1 : 5.60 for the immature cells. SHAPIRO explains the difference in these ratios by germinal selections against lethals hemizygous in the male, and estimates that about 50 percent of the lethals which arise in the immature cells do not reach the spermatozoa stage.

The use of adults with a designated time interval for mature and immature cells leaves some doubt as to the actual stage being treated. The classification of mutants as lethals in this type of test is hard to evaluate since several situations can produce a lethal action. A difference in the number of sex-linked visible mutations recovered by MOORE (1934) for treated adults and larvae gives a better indication of a difference in the 2 stages. By mating to attached-X females, the sex-linked lethals were eliminated. A greater number of viable sex-linked visible mutations were recovered from the mature sperm than from sperm of males which had been treated as larvae.

A comparison of the mutation rates at specific autosomal loci in the mature sperm and spermatogonia shows a lower rate for the latter. For these loci, the confidence interval of 0.728 to 2.79×10^{-8}/r/locus for the spermatogonial rate falls below the values of the confidence interval of 4.64 to 7.34×10^{-8}/r/locus obtained for mature sperm. The data seem to indicate a significant difference in the rates of visible mutations for the 2 stages. This difference in the rates cannot be explained by any kind of germinal selection against mutations in the hemizygous condition since autosomal loci were used. The selection against the hemizygous X in the male may occur but in general the available data from Drosophila do not support germinal selection.

Histological examination of irradiated immature stages of *Pediculus corporis* and *D. melanogaster* by PONTECORVO (1944) indicates that no germinal selection occurs. In Pediculus the meiotic divisions occur early in spermatogenesis so that the germ cells are haploid earlier than in Drosophila. In Pediculus with a dose of 4000 r PONTECORVO reports the following:

"It should be noted that all usual chromosome rearrangements are produced and can be seen (testing of spermatozoa for dominant lethals confirms it): but the cells carrying these rearrangements, many of which are 'lethals,' go through six mitoses and spermateleosis undisturbed. Apart from the direct observation of no cells individually killed, counting the spermatozoa in each bundle (easily done in cross sections) confirms that no germinal selection takes place in all these stages. The only exception is that occasionally a whole

cyst is killed; but this is almost certainly a secondary effect of some injury to nongerminal tissue."

For Drosophila, PONTECORVO continues:

"What histological observation is possible in *Drosophila* confirms that the same situation, *mutatis mutandis*, holds here too. Irradiated testes show necrosis of a small number of apical cells (homologous with the premeiotic stages in the louse) and no necrosis of cells in the spermatogonial divisions, meiosis and spermateleosis, again with the exception of occasional cysts degenerating as a whole."

The data from gynandromorph studies by PATTERSON and STONE (1938) do not show any serious selection during embryological development against aneuploid cells as compared to cells with the normal component of chromosomes. The number of gynandromorphs in each of the detectable classes produced from treated males and carrying an extra fragment of a broken chromosome in the male tissue was as follows: Six were three-fourth male tissue, 25 were one-half male, 13 were one-fourth male, and 5 contained less than one-fourth male tissue. The one-half and three-fourth gynandromorphs could not have been recovered in these relative frequencies if drastic selection had occurred against the aneuploid cells.

A test of tolerance to heterozygous deletions of portions of the third chromosome of *D. melanogaster* was reported by PATTERSON, BROWN and STONE (1940). Individuals hypoploid for different regions of the third chromosome were obtained by segregation from 2 heterozygous translocations which were broken at different points on the chromosome. Some of the hypoploid males survived and were fertile with heterozygous deletions as long as the longest deletions recovered from irradiated sperm in the present experiment. Competition with normal gametes did not eliminate these gametes.

Since dominant Minutes are often produced by small deletions, the recovery of such Minutes from treated spermatogonia in the present experiment indicates the survival of this type of deletion. About one-half the total number of Minutes were recovered from the spermatogonia as groups of two or more from the same tested male; the remaining Minutes were recovered as single individuals from different males. The Minutes from both sperm and spermatogonia were phenotypically similar and were usually weak and semisterile. In a few cases from both tests, they produced a few offspring, some of which showed the dominant Minute effect. In the mature sperm data 96 Minutes were recovered in a sample of 49,512 at 3000 r; in spermatogonia, 106 Minutes were recovered in a sample of 273,574 F_1's at 900 r. In the control tests, 14 Minutes were recovered in a sample of 45,504, with 3 of the 14 recovered from 1 male. Therefore proportion of Minutes to normal would be 1 : 3250 for the controls; 1 : 2580 for spermatogonia at 900 r and 1 : 516 for mature sperm at 3000 r. These two types of data support the assumption that deletions will survive in the immature cells of Drosophila.

Translocations when heterozygous in the males give about 50 percent aneuploid sperm from nondisjunction (BROWN 1940). The aneuploid sperm

produce zygotic mortality as shown by egg counts. The chance of recovering a translocation present heterozygous in spermatogonial cells would be reduced one-half by this zygotic mortality. However, this reduction is not a determinate factor in the present experiment, since a small number of this type of aberration would be expected at 900 r. This possible source of reduction in variants will not account for the difference in the sperm and spermatogonial mutation rates.

In testing the variants from irradiated sperm WARD found that about half the mutations tested had no detectable chromosome aberrations, the other half being composed of deletions, inversions, and translocations (WARD and ALEXANDER 1952, and unpublished). This 1 : 1 ratio at 3000 r would not be expected at 900 r judging from the translocation study on *Drosophila virilis* by BAKER (1949). The frequency-dose relation showed that the percentage of sperm with interchanges increased more rapidly than the first power of the dose for 1000, 2000, and 3000 r of X radiation. On this basis, approximately 0.2 as many two-break rearrangements would be expected with a dose of 900 as with 3000 r. Mutants with no chromosome rearrangements (point mutations) would be expected to increase linearly with dose and 0.3 as many point mutations would be expected at 900 r (OLIVER 1932). The ratio of point mutations to chromosome aberrations should approach a 3 : 2 ratio for 900 r as compared to a 1 : 1 ratio for 3000 r. If the number of position effect mutants calculated on this basis from the mature sperm data is added to the number of spermatogonial mutants, the confidence intervals for the two stages would still not overlap. An average mutation rate can be calculated for mature sperm on point mutations without including the position-effect mutants. With about half the mutants from mature sperm at 3000 r being associated with visible chromosomal abnormalities, the rate of point mutations is about 3.0×10^{-8}/r/locus. Although this is twice the rate of point mutations recovered from spermatogonia, the 95 percent confidence ranges overlap to some degree.

Mutation rates for the mature sperm and the premeiotic spermatogonia show a different amount of genetic damage from X-radiation. The data from different Drosophila studies do not support the assumption that drastic germinal selection occurs. This difference in radiation damage may be due, in part, to the difference in probability of inducing variants caused by chromosomal aberrations. With the present sample size for spermatogonial cells, the absence of such aberrations among the recovered spermatogonial mutants can be considered indicative but not conclusive evidence that they do not occur. LÜNING (1952a) found a difference in rate of breaks measured as dominant lethals and hyperploid males in the various stages of the postmeiotic cells of spermateleosis. This difference was not found in the test for " point mutations " and gynandromorphs. There was a definite drop in the number of hyperploid males, mutations, and gynandromorphs when cells which were treated in meiotic or premeiotic stages were tested. These tests indicate a reduction in the genetic effects of radiation in premeiotic cells but an increase in sensitivity in the spermatid stage even above that of sperm.

A comparable study of mutation rates at specific loci in another species of

Drosophila has been reported by GIRVIN (1949). Seven loci on the X chromosome of *D. virilis* were used in his experiment. When only the proven mutations were counted and the rates for the 7 loci were calculated in the same way as in the present experiment, the following values were obtained: yellow, 4.69×10^{-8}/r; crossveinless, 2.24×10^{-8}; vermilion, 4.64×10^{-8}; singed, 11.34×10^{-8}; dusky, 3.52×10^{-8}; white, 12.67×10^{-8}; apricot, 13.81×10^{-8}/r: average rate for 7 loci, 7.56×10^{-8}/r. A determination of the confidence limits for this average would be difficult since a different number of individuals were checked for some of the loci. This rate would undoubtedly overlap the range of 4.64 to 7.34×10^{-8}/r/locus obtained for the third chromosome of *D. melanogaster* in the present experiment. The average rate of 4.39×10^{-8}/r/locus obtained by PATTERSON for the third chromosome of *D. melanogaster* is also quite similar to the *D. virilis* rate. This comparison of average rates shows no obvious difference in the rates for the 2 species.

In general, the mutation rates at specific loci induced in Drosophila sperm have been found to be lower than the spermatogonial mutation rates reported by RUSSELL (1951) for autosomal loci of the mouse. The mean observed rate for the spermatogonia of the mouse of $(25 \pm 3.7) \times 10^{-8}$/r/locus for 7 autosomal loci is significantly higher than the mean spermatogonial rate of 1.5×10^{-8}/r/locus obtained for 8 autosomal loci of Drosophila. The mean mutation rate for spermatogonia in the mouse is also significantly higher than the mean mate of 5.98×10^{-8}/r/locus obtained for the mature sperm of Drosophila.

The recent demonstration that several more loci are members of pseudoallelic series makes it necessary to consider the effect of pseudoalleles on specific loci studies. Pseudoalleles have been reported for the Star locus by LEWIS (1945), for lozenge by GREEN and GREEN (1949), for vermilion by GREEN (1952, and unpublished), and for white by LEWIS (1952). Very similar cases have been reported for scute and achaete by DUBININ (1933) and by SEREBROVSAÎA (1938). Since a mutation in any one of the component parts of a pseudoallelic series will produce a mutant allele, the relative radiation hazard is still expressed by the mutation rate. However, the number of component parts which are contained in a pseudoallelic series could be reflected in the mutation rates. A locus with several component parts could give a higher mutation rate than a single component locus.

SUMMARY

1. Mutation rates were obtained for 8 specific loci on the third chromosome of *Drosophila melanogaster*. With a dose of 3000 r of X-radiation, the rates for mature sperm varied from 2.7×10^{-8}/r for the thread locus to 8.75×10^{-8}/r for the peach and ebony loci. The 8 loci gave an average rate of 5.98×10^{-8}/r/locus. Of the 58 X-ray-induced mutations, 20 were viable, 35 were lethal, and 3 were semilethal when homozygous.

2. Spermatogonial cells in larvae 20 to 22 hours old were irradiated with 900 r of X-radiation. Single mutant individuals and clusters of the same mutation were recovered from adult males which has been treated as larvae. A variation in the size of the clusters indicated that the number of sperma-

togonia under test varied from 7 to more than 100. The spermatogonial mutation rate is estimated to be approximately 1.52×10^{-8}/r/locus. Four spermatogonial mutants were viable, 3 lethal, and 1 semilethal in the homozygous condition.

3. A lower average mutation rate obtained for spermatogonia than for sperm can be better explained by a differential genetic sensitivity of the 2 stages than by germinal selection.

4. The average mutation rate for the 8 loci of the third chromosome for *D. melanogaster,* from treated sperm, is similar to the average sperm rate obtained by GIRVIN (1949) for the X-chromosome of *D. virilis.* The average spermatogonial rate of 1.5×10^{-8}/r/locus for *D. melanogaster* is significantly lower than the spermatogonial rate of $(25 \pm 3.7) \times 10^{-8}$/r/locus obtained by RUSSELL (1951) for the mouse.

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GENETICS Vol. 39 No. 3 MAY, 1954

CONTENTS OF PRECEDING ISSUES

SEPTEMBER, 1953

KYLE, W. H., and A. B. CHAPMAN, Experimental check of the effectiveness of selection for a quantitative character.

BREWBAKER, JAMES L., Oppositional allelism in diploid and autotetraploid *Trifolium hybridum* L.

WALLACE, BRUCE, and CAROL MADDEN, The frequencies of sub- and supervitals in experimental populations of *Drosophila melanogaster*.

DOBZHANSKY, THEODOSIUS, and BORIS SPASSKY, Genetics of natural populations. XXI. Concealed variability in two sympatric species of Drosophila.

LAUGHNAN, JOHN R., The effect of the *sh*$_2$ factor on carbohydrate reserves in the mature endosperm of maize.

LEVINTHAL, C., and N. VISCONTI, Growth and recombination in bacterial viruses.

DUNN, L. C., and S. GLUECKSOHN-WAELSCH, The failure of a t-allele (t^8) to suppress crossing over in the mouse.

PROUT, TIMOTHY, CHARLES HUEBSCHMAN, HOWARD LEVENE and FRANCIS J. RYAN, The proportions of nuclear types in Neurospora heterocaryons as determined by plating conidia.

NOVEMBER, 1953

GOWEN, JOHN W., JANICE STADLER, H. H. PLOUGH and HELEN N. MILLER, Virulence and immunizing capacity of *Salmonella typhimurium* as related to mutations in metabolic requirements.

SIEGEL, R. W., A genetic analysis of the mate-killer trait in *Paramecium aurelia*, variety 8.

LEVINE, MYRON, The diverse mate-killers of *Paramecium aurelia*, variety 8: their interrelations and genetic basis.

MCCLINTOCK, BARBARA, Induction of instability at selected loci in maize.

STEBBINS, G. L., and FUNG TING PUN, Artificial and natural hybrids in the Gramineae, tribe Hordeae. VI. Chromosome pairing in *Secale cereale* x *Agropyron intermedium* and the problem of genome homologies in the Triticinae.

EATON, ORSON N., Heterosis in the performance of mice.

KAPLAN, WILLIAM D., The influence of Minutes upon somatic crossing over in *Drosophila melanogaster*.

Abstracts of papers presented at the 1953 Meetings of the Genetics Society of America.

JANUARY, 1954

PLOUGH, HAROLD H., Edwin Grant Conklin.

STALKER, HARRISON D., Parthenogenesis in Drosophila.

CASTLE, W. E., Coat color inheritance in horses and in other mammals.

SLATIS, HERMAN M., and DAVID A. WILLERMET, The miniature complex in *Drosophila melanogaster*.

SWAMINATHAN, M. S., Nature of polyploidy in some 48-chromosome species of the genus Solanum, section Tuberarium.

BRNCIC, DANKO, Heterosis and the integration of the genotype in geographic populations of *Drosophila pseudoobscura*.

CAIN, A. J., and P. M. SHEPPARD, Natural selection in Cepaea.

AVERS, CHARLOTTE J., Chromosome behavior in fertile triploid Aster hybrids.

NOVITSKI, EDWARD, The compound X chromosomes in Drosophila.

MARCH, 1954

SCHERR, GEORGE H., MARVIN FISHMAN and R. H. WEAVER, The mutagenicity of some carcinogenic compounds for *Escherichia coli*.

BENNETT, J. H., Panmixia with tetrasomic and hexasomic inheritance.

KRAMER, H. H., ROSEMARIE VEYL and W. D. HANSON, The association of two genetic linkage groups in barley with one chromosome.

LEVINTHAL, CYRUS, Recombination in phage T2: its relationship to heterozygosis and growth.

KALTER, HAROLD, The inheritance of susceptibility to the teratogenic action of cortisone in mice.

NOVITSKI, E., and G. BRAVER, An analysis of crossing over within a heterozygous inversion in *Drosophila melanogaster*.

WALKER, B. E., and E. R. BOOTHROYD, Chromosome numbers in somatic tissues of mouse and man.

STEFFENSEN, DALE, and T. J. ARNASON, Frequency of chromosome aberrations produced by fractional doses of X-rays in Tradescantia.

HANSON, EARL D., Studies on kappa-like particles in sensitives of *Paramecium aurelia*, variety 4.

CALDECOTT, RICHARD S., B. H. BEARD and C. O. GARDNER, Cytogenetic effects of X-ray and thermal neutron irradiation on seeds of barley.

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